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APPLICATION NUMBER: *60/546,540*
FILING DATE: *February 19, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/05066*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.53(b)(2).

Docket Number	UCSC-08805	Type a plus sign (+) inside this box →	+

INVENTOR(s) / APPLICANT(s)			
Last Name	First Name	Middle Initial	Residence (City and Either State or Foreign Country)
Karin **Gao** **Labuda**	**Michael** **Min** **Tord**		**La Jolla, California** **San Diego, California** **Malmo, Sweden**

U.S. PTO 60/546540 02/19/04 021904

TITLE OF THE INVENTION (280 Characters Max.)

Enhancement of Th2-Dependent and Inflammatory Response

CORRESPONDENCE ADDRESS

MEDLEN & CARROLL, LLP
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San Francisco, California 94105
United States of America

ENCLOSED APPLICATION PARTS *(Check All That Apply)*

X	Specification	Number of Pages 97	X	Assignment (unexecuted)
X	Drawing(s)	*Number of Sheets* 14	X	Power of Attorney (unexecuted)
			X	Declaration (unexecuted)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

—	Charge Account No. **08-1290** in the amount of **$80.00. An originally executed duplicate of this transmittal is enclosed for this purpose.**	FILING FEE AMOUNT ($)	**$80.00**
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This invention was made by an agency of the United States Government under a contract with an agency of the United States Government.

— No.

X Yes, the name of the U.S. Government agency and the Government contract number are: National Institutes of Health, number R21AI48542

Respectfully submitted,

Date: February 19, 2004

Mahattamdan

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— Additional inventors are being named on separately numbered sheets attached hereto.

Express Mail Label: **EV 329476559 US**

PATENT
Attorney Docket No.: **UCSD-08805**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Michael Karin, Min Gao, and Tord Labuda

For: **Enhancement of Th2-Dependent and Inflammatory Response**

Mail Stop Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATION UNDER 37 C.F.R. § 1.10

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on **February 19, 2004** , in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. § 1.10, Mailing Label Number **EV 329 476 559 US** addressed to: **Mail Stop Provisional Patent Application,** Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Karen B. Webber

TRANSMITTAL COVER SHEET FOR FILING PROVISIONAL APPLICATION (37 C.F.R. § 1.51(2)(i))

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.53(b)(2).

1. The following comprises the information required by 37 C.F.R. § 1.51(a)(2)(i)(A):

2. The names of the inventor(s) is/are (37 C.F.R. § 1.51(a)(2)(i)(B)):

   Michael Karin
   Min Gao
   Tord Labuda

3. Addresses of the inventor(s), as numbered above (37 C.F.R. § 1.51(a)(2)(i)(C)):

   7710 E. Roseland Drive, La Jolla, CA 92037
   8223 Jade Coast Road #119, San Diego, CA 92126
   Sodra Forstadsgatan 19A, 21143 Malmo, Sweden

4. The title of the invention is (37 C.F.R. § 1.51(a)(2)(i)(D)):

   Enhancement of Th2-Dependent and Inflammatory Response

5. The name, registration, and telephone number of the attorney (*if applicable*) is (37 C.F.R. § 1.51(a)(2)(i)(E)):

Maha A. Hamdan
Reg. No.: 43,655
Tel.: 415.904.6500

(complete the following, if applicable)

X A Power of Attorney (unexecuted) accompanies this cover sheet.

6. The docket number used to identify this application is (37 C.F.R. § 1.51(a)(2)(i)(F)):

Docket No.: **UCSD-08805**

7. The correspondence address for this application is (37 C.F.R. § 1.51(a)(2)(i)(G)):

MEDLEN & CARROLL, LLP
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San Francisco, California 94105

8. Statement as to whether invention was made by an agency of the U.S. Government or under contract with an agency of the U.S. Government. (37 C.F.R. § 1.51(a)(2)(i)(H)):

This invention was made by an agency of the United States Government, or under contract with an agency of the United States Government.

___ No.

X Yes.

The name of the U.S. Government agency and the Government contract number are: National Institutes of Health, number R21AI48542.

9. Identification of documents accompanying this cover sheet:

A. Documents required by 37 C.F.R. § 1.51(a)(2)(ii)-(iii):

Specification: No. of pages **_97_**

Drawings: No. of sheets **_14__** (informal)

B. Additional documents:

___ Claims: No. of claims

X Power of Attorney (unexecuted)

X Assignment (unexecuted)

X Declaration (unexecuted)

Express Mail Label: **EV 329476559 US**

PATENT
Attorney Docket No.: **UCSD-08805**

10. Fee

The filing fee for this provisional application, as set in 37 C.F.R. § 1.16(k), is $160.00, for other than a small entity, and $80.00, for a small entity.

X Applicant is a small entity.

11. Small Entity Statement

X This is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27.

12. Fee payment being made at this time

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Date: February 19, 2004

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ENHANCEMENT OF Th2-DEPENDENT AND INFLAMMATORY RESPONSE

GOVERNMENT INTERESTS

The present invention was funded by grants from the National Institutes of Health, number R21AI48542. As such, the United States Government may have certain rights to this invention.

FIELD OF THE INVENTION

The present invention relates to altering the levels of Th2 cytokine production, and in particular, biasing the cytokine expression profile towards Th2 cytokine production through mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1), the screening of agents that increase Th2 cytokine production, and the treatment of Th1 associated autoimmune diseases *in vivo*. In one embodiment, the present invention relates to agents including but not limited to reducing the activity of MEKK1, leading to increased levels of Th2 cytokine production.

BACKGROUND

Debilitating autoimmune disorders are associated with chronically activated T cell populations producing Type 1 pro-inflammatory cytokines (Th1 populations). These autoimmune disorders are classified as Th1 or type 1 disorders and include type 1 diabetes, autoimmune thyroiditis, *etc.* One therapeutic mechanism is to treat these disorders with cytokines such as IL-10, IL-4, and IL-11 typically considered type 2 cytokines produced by T cells categorized as but not limited to Th2 populations. *In vitro*, the presence of Th2 cytokines may counter or reduce the production of type 1 cytokines in addition to inhibiting further Th1 maturation or biasing further maturation of T cells towards Th2 cytokine production. However, although these cytokine treatments show promise for certain diseases, these treatments are expensive and require frequent injections or infusions.

Increased cytokine production occurs when T cell receptor (TCR) engagement induces activation of several transcription factors, including AP-1 that in turn activates a related network of signal transduction molecules including numerous MAP kinases, JNK molecules

and ubiqases. The intensity or duration of T cell activation can bias the cytokine expression profile towards Th1 like or Th2 like, but the underlying biochemical mechanism is unknown.

Thus, there is a need to determine the underlying biochemical mechanism for augmenting Th2 cytokine production in order to find new ways to identify drugs that will reduce chronically activated Th1 cell populations, and in particular augment signaling pathways that produce Th2 cytokines.

SUMMARY OF THE INVENTION

The present invention relates to altering the levels of Th2 cytokine production, and in particular, biasing the cytokine expression profile towards Th2 cytokine production through mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1), the screening of agents that increase Th2 cytokine production, and the treatment of Th1 associated autoimmune diseases *in vivo*. In one embodiment, the present invention relates to agents including but not limited to reducing the activity of MEKK1, leading to increased levels of Th2 cytokine production.

In one embodiment, the prevention and treatment of diseases known to be associated with high T helper 1 (Th1) cell cytokine levels is accomplished by interfering with the MEKK1-ITCH cascade pathway and interactions as described herein to increase the levels of T helper 2 (Tc2) cytokines. In one embodiment, the prevention and treatment of diseases known to be associated with Th1 cell levels is accomplished by interfering with the MEKK1-ITCH cascade pathway and interactions as described herein to increase the production of Th2 cells. In certain embodiments, the importance of MEKK1-ITCH cascade pathway and the MEKK1-ITCH interactions with the use of MEKK1 and JNK knockout mouse models and the use of JNK and other inhibitors are described. These embodiments serve to distinguish agents that would be drug candidates as anti-inflammatory for treatment of Th1 cytokine disorders.

In one embodiment, the invention provides a method for identifying a test agent as reducing the level of differentiation of T cells into T helper type 1 (Th1) cells comprising reducing mitogen-activated protein kinase/ERK kinase kinase 1 catalytic activity in said T cells. In another embodiment, the invention provides a method for identifying a test agent wherein reducing of said mitogen-activated protein kinase/ERK kinase kinase 1 catalytic

activity comprises increasing the level of differentiation of said T cells into T helper type 2 (Th2) cells. In another embodiment, the invention provides a method for identifying a test agent wherein the reducing of said mitogen-activated protein kinase/ERK kinase kinase 1 catalytic activity comprises increasing the level of one or more T helper type 2 (Th2) cell cytokine that is produced by said T cell. In another embodiment, the invention provides a method for identifying a test agent wherein the increased level of said Th2 cytokine occurs in the absence of an increase in the level of one or more T helper type 1 (Th1) cytokine. In another embodiment, the invention provides a method for identifying a test agent wherein said Th1 cytokine is chosen from one or more of interferon-gamma and Interleukin-2. In another embodiment, the invention provides a method for identifying a test agent wherein said Th2 cytokine is chosen from one or more of Interleukin-4, Interleukin-5, Interleukin-10, and Interleukin-13. In another embodiment, the invention provides a method for identifying a test agent wherein said increasing the level of said Th2 cell cytokine comprises increasing the level of mRNA encoding said Th2 cytokine. In another embodiment, the invention provides a method for identifying a test agent wherein said mRNA encoding said Th2 cytokine is increased 5 fold. In another embodiment, the invention provides a method for identifying a test agent wherein said reducing of said mitogen-activated protein kinase/ERK kinase kinase 1 catalytic activity comprises increasing the level of proliferation of T helper type 2 (Th2) cells that differentiate from said T cells. In another embodiment, the invention provides a method for identifying a test agent wherein said reducing of said mitogen-activated protein kinase/ERK kinase kinase 1 catalytic activity comprises introducing a mutation in the gene encoding mitogen-activated protein kinase/ERK kinase kinase 1. In another embodiment, the invention provides a method for identifying a test agent wherein said T cells comprise thymocyte cells. In another embodiment, the invention provides a method for identifying a test agent wherein said T cells comprise splenocyte cells. In another embodiment, the invention provides a method for identifying a test agent wherein said T cells are *in vitro*. In another embodiment, the invention provides a method for identifying a test agent wherein said T cells are *in vivo* in an animal. In another embodiment, the invention provides a method for identifying a test agent wherein said animal is human. In another embodiment, the invention provides a method for identifying a test agent wherein said human is chosen from a human

that is: (a) suspected of having a Th1 - mediated disease; (b) not suspected of having a Th1 - mediated disease; (c) suspected of being capable of developing a Th1 - mediated disease; and (d) suspected of not being capable of developing a Th1 - mediated disease. In another embodiment, the invention provides a method for identifying a test agent wherein said Th1-mediated disease is chosen from multiple sclerosis, type 1 diabetes, autoimmune thyroiditis, and rheumatoid arthritis.

In one embodiment, the present invention provides a method for identifying a test agent as reducing the level of differentiation of T cells into T helper type 1 (Th1) cells, comprising: a) providing: i) a test agent; and ii) mitogen-activated protein kinase/ERK kinase kinase 1; and b) contacting said test agent and said mitogen-activated protein kinase/ERK kinase kinase 1; and c) detecting reduced mitogen-activated protein kinase/ERK kinase kinase 1 kinase activity in the presence of said agent compared to in the absence of said agent, thereby identifying said test agent as causing one or more of increasing Th2 cells, decreasing the level of Th1 cells, and decreasing Th1 disease. In another embodiment, the invention provides a method for identifying a test agent wherein said method comprises one or more of: (a) identifying said agent as increasing the level of differentiation of said T cells into T helper type 2 (Th2) cells; (b) identifying said agent as increasing the level of one or more T helper type 2 (Th2) cell cytokine that is produced by said T cell; and (c) identifying said agent as increasing the level of proliferation of T helper type 2 (Th2) cells that differentiate from said T cells.

In one embodiment, the present invention provides a method for increasing Th2 cytokine levels produced by T cells, comprising: (a) providing: (i) an inhibitor of E3 ubiquitin ligase itch; (ii) T cells; (iii) test agent; and (b) contacting said T cells in the presence of said test agent to produce contacted T cells and in the absence of said test agent to produce control T cells; and (c) detecting reduced activity of E3 ubiquitin ligase itch in said contacted T cells compared to E3 ubiquitin ligase itch in said control T cells, wherein said detecting identifies said test agent as increasing Th2 cytokine levels produced by T cells.

In one embodiment, the present invention provides a method for increasing Th2 cytokine levels produced by T cells, comprising: (a) providing: (i) a kinase inhibitor, wherein said kinase is one or more of mitogen-activated protein kinase/ERK kinase kinase 1

and C-Jun N-terminal kinase 1; (ii) T cells; (iii) test agent; and (b) contacting said T cells in the presence of said test agent to produce contacted T cells and in the absence of said test agent to produce control T cells; and (c) detecting reduced activity of said kinase in said contacted T cells compared to said kinase in said control T cells, wherein said detecting identifies said test agent as increasing Th2 cytokine levels produced by T cells. In another embodiment, the invention provides a method further comprising, (d) identifying said test agent as increasing the level of one or more of Th2 cytokine. In another embodiment, the invention provides a method wherein said Th2 cytokine is one or more of Interleukin-4, Interleukin-5, Interleukin-10, and Interleukin-13. In another embodiment, the invention provides a method further comprising, (d) identifying said test agent as decreasing the level of Th1 cytokines. In another embodiment, the invention provides a method wherein said kinase inhibitor comprises SB600125.

In one embodiment, the present invention provides a method for increasing Th2 cytokine levels produced by T cells, comprising reducing the activity of a E3 ubiquitin ligase itch.

In one embodiment, the present invention provides a method for increasing Th2 cytokine levels produced by T cells, comprising reducing the activity of a mitogen-activated protein kinase/ERK kinase kinase 1.

In one embodiment, the present invention provides a method for increasing Th2 cytokine levels produced by T cells, comprising reducing the activity of a C-Jun N-terminal kinase 1.

In one embodiment, the present invention provides a method for increasing Th2 cytokine levels produced by T cells, comprising: (a) providing: (i) T cells; and (ii) agent that reduces activity of E3 ubiquitin ligase itch; and (b) contacting said T cells with said agent under conditions such that said agent reduces said activity of said E3 ubiquitin ligase itch.

In one embodiment, the present invention provides a method for increasing Th2 cytokine levels produced by T cells, comprising: (a) providing: (i) T cells; and (ii) agent that reduces activity of a kinase, wherein said kinase is one or more of mitogen-activated protein kinase/ERK kinase kinase 1 and C-Jun N-terminal kinase 1; and (b) contacting said T cells with said agent and without said agent under conditions such that said agent reduces activity

of said kinase. I n another embodiment, the invention provides a method for increasing Th2 cytokine levels produced by T cells further comprising (c) identifying said test agent as comprising increasing the level of Interleukin-10 produced by said T cells. In another embodiment, the invention provides a method for increasing Th2 cytokine levels produced by T cells wherein said T cells are inflammatory disease T cells. In another embodiment, the invention provides a method for increasing Th2 cytokine levels produced by T cells wherein said inflammatory disease is one or more of type 1 diabetes, autoimmune thyroiditis, multiple sclerosis and rheumatoid arthritis.

In one embodiment, the present invention provides a method for increasing Th2 cytokine levels produced by pro-inflammatory disease T cells, comprising: (a) providing: (i) pro-inflammatory disease T cells; and (ii) agent that reduces activity of E3 ubiquitin ligase itch; and (b) contacting said pro-inflammatory disease T cells with said agent under conditions such that said agent increases the level of Interleukin-10 produced by said T cells.

In one embodiment, the present invention provides a method for reducing inflammation and disease associated with Th1 cell abundance by increasing the *in vivo* production of Th2 cells comprising reducing one or more of MEKK1 - MEKK4/MEKK7 - JNK - ITCH cascade pathway activity and direct MEKK1-ITCH protein to protein interactions. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein said reducing is comprises using one or more MEKK1 enzyme inhibitors. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein said enzyme inhibitors comprises SP600125. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein said reducing comprises using one or more neutralizing antibodies that specifically bind to MEKK1. In another embodiment, the present invention provides a method to reduce inflammation and diseases associated with Th1 cell abundance wherein said reducing is achieved by reducing expression of the MEKK1 gene. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein said reducing comprises using one or more MEKK4/MEKK7 enzyme inhibitors. In another embodiment, the present invention provides a method to reduce

inflammation and disease associated with Th1 cell abundance wherein said MEKK4/MEKK7 enzyme inhibitors comprises SP600125. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein said reducing comprises neutralizing antibodies that specifically bind to MEKK4/MEKK7. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein said reducing is comprises inhibiting expression of the MEKK4/MEKK7 gene. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein said reducing comprises using ITCH enzyme inhibitors. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein said reducing comprises using neutralizing antibodies that specifically bind to ITCH. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein said reducing comprises inhibiting the expression of the *itch* gene. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein said inhibitor comprises SP600125. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein the expression of the JNK gene is suppressed by the use of one or more of RNAi, and antisense molecules. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein the expression of the MEKK1 gene is suppressed by the use of one or more of RNAi and antisense molecules. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein the expression of the MEKK4/MKK7 gene is suppressed by the use of one or more of RNAi and antisense molecules. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein the expression of the Itch gene is suppressed by the use of one or more of RNAi and antisense molecules. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance by increasing the *in vivo* production of Th2 cells wherein the neutralizing antibody is chosen from human antibody and

humanized antibody that invoke minimum and therapeutically acceptable level of immunogenic defense response in a human. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance by increasing the *in vivo* production of Th2 cells wherein the MEKK1 - Itch interactions are reduced by using SP600125. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance wherein MEKK1 - Itch interactions are reduced by the use of neutralizing antibodies against one or more of MEKK1 and Itch. In another embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance by increasing the *in vivo* production of Th2 cells wherein the neutralizing antibody is chosen from human antibody and humanized antibody that invoke minimum and therapeutically acceptable level of immunogenic defense response in a human.

In one embodiment, the present invention provides a composition comprising a transgenic mouse that comprises MEKK1-/MEKK1- or MEKK1-/MEKK1+.

In one embodiment, the present invention provides a method for identifying therapeutic agents that are useful in reducing one or more of MEKK1 - MEKK4/MEKK7 - JNK - ITCH cascade pathway activity and direct MEKK1-ITCH protein to protein interactions comprising: (a) providing; WT and $Mekk1^{KD}$ thymocytes stimulated with anti-CD3 and anti-CD28 for 24 hrs in the absence or presence (0.5 mM) of a JNK inhibitor; (b) preparing cell lysates from said thymocytes; (c) immunoblotting said lysates; and (d) determining levels of one or more of Itch, c-Jun and JunB to identify therapeutic agents that are useful in reducing cascade pathway activity.

In one embodiment, the present invention provides a method to reduce inflammation and disease associated with Th1 cell abundance by increasing the *in vivo* production of Th2 cells. In another embodiment, the present invention provides a method wherein said JNK inhibitor comprises SP600125. In another embodiment, the present invention provides a method for reducing inflammation and disease associated with Th1 cell abundance by increasing the *in vivo* production of Th2 cells wherein said disease is chosen from multiple sclerosis, type 1 diabetes, autoimmune thyroiditis, and rheumatoid arthritis

In one embodiment, the present invention provides a method for reducing the symptoms of type 1 diabetes by increasing *in vivo* production of Th2 cells comprising reducing one or more of MEKK1 - MEKK4/MEKK7 - JNK - ITCH cascade pathway activity and direct MEKK1-ITCH protein to protein interactions.

In one embodiment, the present invention provides a method for reducing the symptoms of autoimmune thyroiditis by increasing *in vivo* production of Th2 cells comprising reducing one or more of MEKK1 - MEKK4/MEKK7 - JNK - ITCH cascade pathway activity and direct MEKK1-ITCH protein to protein interactions.

In one embodiment, the present invention provides a method for reducing the symptoms of multiple sclerosis by increasing *in vivo* production of Th2 cells comprising reducing one or more of MEKK1 - MEKK4/MEKK7 - JNK - ITCH cascade pathway activity and direct MEKK1-ITCH protein to protein interactions.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows exemplary embodiments demonstrating hyperproliferation of $Mekk1^{KD}$ mutant T cells.

Figure 2 shows exemplary embodiments demonstrating the role of reduced JNK activity results in increased thymocyte proliferation.

Figure 3 shows an exemplary embodiment demonstrating enhanced Th2 cytokine production by $Mekk1^{KD}$ thymocytes.

Figure 4 shows an exemplary embodiment demonstrating upregulation of JunB and c-Jun protein levels in $Mekk1^{KD}$ thymocytes.

Figure 5 shows an exemplary embodiment demonstrating MEKK1 promotes ubiquitin-dependent degradation of c-Jun and JunB.

Figure 6 shows an exemplary embodiment demonstrating MEKK1/JNK signaling pathway regulates Itch and Jun turnover.

Figure 7 shows an exemplary embodiment demonstrating MEKK1/JNK signaling pathway negatively and positively regulates JunB stability and activity in response to T cell stimulation.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

As used herein including within this specification and the appended claims, the forms "a," "an" and "the" includes both singular and plural references unless the content clearly dictates otherwise.

As used herein, the term "or" when used in the expression "A or B," and where A and B refer to a composition, disease, product, etc., means one, or the other, or both.

As used herein, the term "comprising" when placed before the recitation of steps in a method means that the method encompasses one or more steps that are additional to those expressly recited, and that the additional one or more steps may be performed before, between, and/or after the recited steps. For example, a method comprising steps a, b, and c encompasses a method of steps a, b, x, and c, a method of steps a, b, c, and x, as well as a method of steps x, a, b, and c. Furthermore, the term "comprising" when placed before the recitation of steps in a method does not (although it may) require sequential performance of the listed steps, unless the content clearly dictates otherwise. For example, a method comprising steps a, b, and c encompasses, for example, a method of performing steps in the order of steps a, c, and b, the order of steps c, b, and a, and the order of steps c, a, and b, *etc.*

As used herein, the term "inflammation" refers to a response of redness, swelling, pain, and a feeling of heat in certain areas that includes but is not limited to an inflammatory response. Inflammation can include but not be limited to characteristics of increased blood flow and entry of leukocytes into the tissues, resulting in swelling, redness, elevated temperature and pain.

As used herein, "pro-inflammatory" and "inflammatory" refers to but is not limited to cells, molecules, signaling pathways, *etc.* that induce and support immune responses that are not limited to and include T1 responses.

As used herein, the term "inflammatory cells" refers to a collection of immune system cells and molecules that invade tissues and organs as part of an immune system response.

As used herein, "immune response" refers to one or more of cell-mediated, humoral and the like. As used herein, "cell mediated response" refers to an immune response that

predominantly involves immune cell activation. As used herein, "humoral" and "humoral response" refers to an immune response that predominately involves antibody production.

In one embodiment, the agent that reduces activity of mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1), C-Jun N-terminal kinase 1 (JNK1), itch, *etc.*, is an antibody, such as MEKK1 or JNK1 or ITCH or MEKK1/MEKK7 complex or MEKK1/MEEK4/MEEK1 complex peptide antibody, and/or MEKK1 or JNK1 or ITCH sequence antibody. The terms "antibody" and "immunoglobulin" are interchangeably used to refer to a glycoprotein or a portion thereof (including single chain antibodies), which is evoked in an animal by an immunogen and which demonstrates specificity to the immunogen, or, more specifically, to one or more epitopes contained in the immunogen. The term "antibody" includes polyclonal antibodies, monoclonal antibodies, blocking antibodies, neutralizing antibodies, inhibiting antibodies, stimulating antibodies, naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof, including, for example, Fab, $F(ab')_2$, Fab fragments, Fd fragments, and Ev fragments of an antibody, as well as a Fab expression library. It is intended that the term "antibody" encompass any immunoglobulin (*e.g.*, IgG, IgM, IgA, IgE, IgD, *etc.*) obtained from any source (*e.g.*, humans, rodents, non-human primates, caprines, bovines, equines, ovines, *etc.*). The term "polyclonal antibody" refers to an immunoglobulin produced from more than a single clone of plasma cells; in contrast "monoclonal antibody" refers to an immunoglobulin produced from a single clone of plasma cells. Monoclonal and polyclonal antibodies may or may not be purified. For example, polyclonal antibodies contained in crude antiserum may be used in this unpurified state.

An example of a MAP kinase kinase antibody, herein incorporated by reference in United States Patent 6,465,618, Nishida, *et al.* (October 15, 2002).

Naturally occurring antibodies may be generated in any species including murine, rat, rabbit, hamster, human, and simian species using methods known in the art. Non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting

of variable heavy chains and variable light chains as previously described [Huse *et al.*, Science 246:1275-1281 (1989)]. These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward *et al.*, Nature 341:544-546 (1989); Hilyard *et al.*, Protein Engineering: A practical approach (IRL Press 1992); and Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995).

Those skilled in the art know how to make polyclonal and monoclonal antibodies which are specific to a desirable polypeptide. For the production of monoclonal and polyclonal antibodies, various host animals can be immunized by injection with the peptide corresponding to any molecule of interest in the present invention, including but not limited to rabbits, mice, rats, sheep, goats, chickens, *etc.* In one preferred embodiment, the peptide is conjugated to an immunogenic carrier (*e.g.*, diphtheria toxoid, bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH)). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward molecules of interest in the present invention, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used (*See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, NY). These include but are not limited to the hybridoma technique originally developed by Köhler and Milstein (Köhler and Milstein, Nature 256:495-497 [1975]), as well as the trioma technique, the human B-cell hybridoma technique (*See e.g.*, Kozbor *et al.* Immunol. Today 4:72 [1983]), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al., in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 [1985]). In some particularly preferred embodiments of the present invention, the present invention provides monoclonal antibodies of the IgG class.

In additional embodiments of the invention, monoclonal antibodies can be produced in germ-free animals utilizing technology such as that described in PCT/US90/02545. In addition, human antibodies may be used and can be obtained by using human hybridomas (Cote *et al.*, Proc. Natl. Acad. Sci. U.S.A.80:2026-2030 [1983]) or by transforming human B cells with EBV virus in vitro (Cole *et al.*, *in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96 [1985]).

Furthermore, techniques described for the production of single chain antibodies (*See e.g.*, U.S. Patent 4,946,778; herein incorporated by reference) can be adapted to produce single chain antibodies that specifically recognize a molecule of interest (*e.g.*, at least a portion of an AUBP or mammalian exosome, as described herein). An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse *et al.*, Science 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a particular protein or epitope of interest (*e.g.*, at least a portion of an AUBP or mammalian exosome).

The invention also contemplates humanized antibodies. Humanized antibodies may be generated using methods known in the art, including those described in U.S. Patent Numbers 5,545,806; 5,569,825 and 5,625,126, the entire contents of which are incorporated by reference. Such methods include, for example, generation of transgenic non-human animals which contain human immunoglobulin chain genes and which are capable of expressing these genes to produce a repertoire of antibodies of various isotypes encoded by the human immunoglobulin genes.

According to the invention, techniques described for the production of single chain antibodies (U.S. 4,946,778; herein incorporated by reference) can be adapted to produce specific single chain antibodies as desired. An additional embodiment of the invention utilizes the techniques known in the art for the construction of Fab expression libraries (Huse *et al.*, Science, 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibody fragments that contain the idiotype (antigen binding region) of the antibody molecule can be generated by known techniques. For example, such fragments include but

are not limited to: the F(ab')2 fragment that can be produced by pepsin digestion of an antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of an F(ab')2 fragment, and the Fab fragments that can be generated by treating an antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art (*e.g.*, radioimmunoassay, ELISA [enzyme-linked immunosorbent assay], "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays [*e.g.*, using colloidal gold, enzyme or radioisotope labels], Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays, *etc.*), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, *etc.*

In an alternative embodiment, the agent that alters the level of binding of MEKK1 or MEKK4 or MKK7 or JNK1 or itch with a MEKK1 or MEKK4 or MKK7 or JNK1 or itch sequence, respectively, is a nucleic acid sequence. The terms nucleic acid sequence therein refer to two or more nucleotides which are covalently linked to each other. Included within this definition are oligonucleotides, polynucleotide, and fragments or portions thereof, DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand. Nucleic acid sequences which are particularly useful in the instant invention include, without limitation, antisense sequences and ribozymes. In an example herein incorporated by reference, Flavell *et al.* August 21, 2003 United States Patent Application 20030157539 A1, a nucleic acid inhibitor comprising IRAK-M reduces toll-like receptor signaling.

In one embodiment, the agent that alters the level of MEKK1 or MEKK4 or MKK7 or JNK1 or itch with a MEKK1 or MEKK4 or MKK7 or JNK1 or itch sequence, is an antisense nucleic acid sequence. Antisense sequences have been successfully used to inhibit the expression of several genes [Markus-Sekura (1988) Anal. Biochem. 172:289-295; Hambor *et al.* (1988) J. Exp. Med. 168:1237-1245; and patent EP 140 308], including the gene encoding VCAM1, one of the integrin $\alpha 4\beta 1$ ligands [U.S. Patent No. 6,252,043, incorporated in its entirety by reference]. As an example, in U.S. patent no. 6,054,440 Monia, *et al.* (April 25,

2000), herein incorporated by reference, antisense sequences were used to modulate Jun N-terminal Kinase Kinase-2 expression through function of nucleic acid molecules encoding Jun N-terminal Kinase Kinase-2. The terms "antisense DNA sequence" and "antisense sequence" as used herein interchangeably refer to a deoxyribonucleotide sequence whose sequence of deoxyribonucleotide residues is in reverse 5' to 3' orientation in relation to the sequence of deoxyribonucleotide residues in a sense strand of a DNA duplex. A "sense strand" of a DNA duplex refers to a strand in a DNA duplex which is transcribed by a cell in its natural state into a "sense mRNA." Sense mRNA generally is ultimately translated into a polypeptide. Thus, an "antisense DNA sequence" is a sequence which has the same sequence as the non-coding strand in a DNA duplex, and which encodes an "antisense RNA" (*i.e.*, a ribonucleotide sequence whose sequence is complementary to a "sense mRNA" sequence). The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.*, "positive") strand. Antisense RNA may be produced by any method, including synthesis by splicing an antisense DNA sequence to a promoter which permits the synthesis of antisense RNA. The transcribed antisense RNA strand combines with natural mRNA produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation, or promote its degradation.

Antisense oligonucleotide sequences may be synthesized using any of a number of methods known in the art (such as solid support and commercially available DNA synthesizers, standard phosphoramidate chemistry techniques, and commercially available services, *e.g.*, Genta, Inc.).

As used herein, the term "RNAi" and "RNA interference" refers to the ability of double stranded RNA to suppress the expression of a gene corresponding to its own sequence.

In some alternative embodiments, the agent that alters the level of T2 cytokines or MEKK1 or MEKK4 or MKK7 or JNK1 or itch and/or T1 cytokines or MEKK1 or MEKK4 or MKK7 or JNK1 or itch is a ribozyme nucleic acid sequence. Ribozyme sequences have been successfully used to inhibit the expression of several genes including the gene encoding VCAM1, which is one of the integrin $\alpha 4\beta 1$ ligands [U.S. Patent No. 6,252,043, incorporated in its entirety by reference]. The term "ribozyme" refers to an RNA sequence that hybridizes

to a complementary sequence in a substrate RNA and cleaves the substrate RNA in a sequence specific manner at a substrate cleavage site. Typically, a ribozyme contains a "catalytic region" flanked by two "binding regions." The ribozyme binding regions hybridize to the substrate RNA, while the catalytic region cleaves the substrate RNA at a "substrate cleavage site" to yield a "cleaved RNA product." Examples of ribosomes that modulate genes related to apoptosis are NF-KappaB genes, such as REL-A, REL-B, REL (c-rel), NFKB1 (p105/p50) and NFKB2 (p100)/p52/p49), herein incorporated by reference, are demonstrate in United States Patent Application, 20020177568 A1, Stinchcomb, *et al.*, November 28, 2002.

Molecules which find use as agents for specifically altering the level of specific binding of MEKK1 or MEKK4 or MKK7 or JNK1 or itch with effector molecule sequences include organic molecules, inorganic molecules, and libraries of any type of molecule, which can be screened using a method of the invention, and which may be prepared using methods known in the art. These agents are made by methods for preparing oligonucleotide libraries [Gold *et al.*, U.S. Patent No. 5,270,163, incorporated by reference]; peptide libraries [Koivunen *et al.* J. Cell Biol., 124: 373-380 (1994)]; peptidomimetic libraries [Blondelle *et al.*, Trends Anal. Chem. 14:83-92 (1995)] oligosaccharide libraries [York *et al.*, Carb. Res. 285:99-128 (1996) ; Liang *et al.*, Science 274:1520-1522 (1996); and Ding *et al.*, Adv. Expt. Med. Biol. 376:261-269 (1995)]; lipoprotein libraries [de Kruif *et al.*, FEBS Lett., 399:232-236 (1996)]; glycoprotein or glycolipid libraries [Karaoglu *et al.*, J. Cell Biol. 130:567-577 (1995)]; or chemical libraries containing, for example, drugs or other pharmaceutical agents [Gordon *et al.*, J. Med. Chem. 37:1385-1401 (1994); Ecker and Crook, Bio/Technology 13:351-360 (1995), U.S. Patent No. 5,760,029, incorporated by reference]. Libraries of diverse molecules also can be obtained from commercial sources.

As used herein, the term "T cell" refers to lymphocytes that differentiate primarily in the thymus and are central to the control and development of immune responses. Examples of T cells include but are not limited to T helper cells (*e.g.* Th0, Th1 and Th2 and the like) and cytotoxic T cells (*e.g.* Tc1 and Tc2 and the like), killer T cells (*e.g.* natural killer T cells (NKT cells), cytotoxic T cells, and the like), naive T cells and the like.

As used herein, the terms "CD," "CD antigen," "Cluster of differentiation" refers to a designation assigned to leukocyte cell surface molecules. For example, "CD3" refers to but is not limited to a trimeric complex of g, d and e chains which together with a zz homodimer or zh heterodimer acts as a signal transducing unit for the T-cell receptor. Further, each CD molecule is identified by a given group of monoclonal antibodies. For example, "anti-CD3" and "antiCD3" and in certain situations "CD3" refers to antibodies that bind to CD3 molecules. For another example, "anti-CD28" and "antiCD28" and in certain situations "CD28" refers to antibodies that bind to CD28 molecules. As used herein, the term "CD4" refers to a cell surface glycoprotein, found on helper T-cells, that are referred to as CD4 T cells. CD4 can function to recognizes MHC class II molecules on antigen-presenting cells. As used herein, the term "CD8" refers to a cell surface glycoprotein, found on cytotoxic T-cells. CD8 can function to recognize MHC class I molecules on target cells.

As used herein, the terms "Th1 disease,""Th1 disorder," "type 1 autoimmune disease," "type 1 autoimmune disorder" refers to a disorder wherein the immune response comprises a cellular immune response (*e.g.* T cell activation associated with IFN-gamma) that is more prominent than a humoral response (*e.g.* T cell activation associated with IL-4, B cell activation and antibody production). As used herein, the terms "Th1 cytokine response"and "T1 cytokine response" refers to an immune response whose most prominent feature comprises abundant CD4 helper T cell activation that is associated with increased levels of T1 cytokines (*e.g.* interferon-gamma, *etc.*) relative to these cytokine amounts in the absence of activation. A T1 cytokine response can also refer to the production of T1 cytokines from other white blood cells and nonwhite blood cells. A Th1 cytokine response can include abundant CD8 cytotoxic T lymphocyte activity including T1 cytokine production, referred to as Tc1. A Th1 response is typically promoted by CD4 "Th1" T-helper cells however a Th1 response can include CD8 Tc1 T cytotoxic cells.

As used herein, the terms "Type 1 cytokines," "Th1 cytokines," "Tc1 cytokines," "T1 cytokines," refers to cytokines that include but are not limited to IL-1, IL-2, IL-8, IL-12, IL-18, interferon-gamma, tumor necrosis factor-alpha (TNF-alpha), *etc.* Conversely, T1 cytokine production is not limited to T1 mediated diseases and disorders since increased levels of T1 cytokines might be found during certain stages of diseases and disorders classified as T2.

Examples of T1 disorders include but are not limited to multiple sclerosis, type 1 autoimmune thyroid disorders,

As used herein, the terms "type 1 diabetes mellitus"and "insulin dependent diabetes" refers to a disorder following an immune system mediated loss of insulin from autoimmune destruction of the insulin-producing cells of the pancreas.

As used herein, the term "multiple sclerosis" refers to a chronic neurologic disease of the central nervous system (CNS). MS is classified but not limited to a demyelinating and an axonal disease. Multiple Sclerosis is a disease in which the immune system targets nerve tissues of the central nervous system. Most commonly, damage to the central nervous system occurs intermittently, allowing a person to lead a fairly normal life. At the other extreme, the symptoms may become constant, resulting in a progressive disease with possible blindness, paralysis,

As used herein, the term "rheumatoid arthritis" refers to arthritic symptoms wherein the immune system is presumed to predominantly target the lining (synovium) that covers various joints thus causing pain, swelling, and stiffness of the joints. Although antibodies are believed to have a role, the damage to the synovial lining is believed to be caused through cell-mediated inflammation and type 1 cytokine damage.

As used herein, the term "type 1 autoimmune thyroid disorder" refers to thyroid disorders that include but are not limited to Hashimoto's thyroiditis. As used herein, the term "Hashimoto's thyroiditis" refers to symptoms in human patients of hypoactive thyroid function that can be but is not limited to immune system destruction of thyroid tissue (e.g. excess type 1 cytokine, killer T cells whose activation destroys thyroid cells and tissues, *etc.*). In certain patients, circulating T cells produce higher levels of type 1 cytokines when compared to healthy people.

As used herein, the terms "Th2 disease,""Th2 disorder," "type 2 autoimmune disease," "type 2 autoimmune disorder" refers to a disease or disorder wherein the immune response comprises a humoral response (*e.g.* T cell activation associated with IL-4, B cell activation and antibody production) that is more prominent than acellular immune response (*e.g.* T cell activation associated with IFN-gamma). As used herein, the terms "Th2 cytokine response"and "T2 cytokine response" refers to an immune response whose most prominent

feature comprises abundant CD4 helper T cell activation that is associated with increased levels of T2 cytokines (*e.g.* IL-4, *etc.*) relative to these cytokine amounts in the absence of activation. A T2 cytokine response can also refer to the production of T2 cytokines from other white blood cells and nonwhite blood cells. A Th2 cytokine response can include abundant CD8 cytotoxic T lymphocyte activity including T2 cytokine production, referred to as Tc2 responses. A Th1 response is typically promoted by CD4 "Th1" T-helper cells however a Th2 response can include CD8 Tc2 T cytotoxic cells.

As used herein, the terms "Type 2 cytokines," "Th2 cytokines," "Tc2 cytokines," "T2 cytokines," refers to cytokines that include but are not limited to IL-4, IL-5, IL-6, IL-10, IL-13, IL-15, *etc.* Conversely, T2 cytokine production is not limited to T2 mediated diseases and disorders since increased levels of T2 cytokines might be found during certain stages of diseases and disorders classified as T1.

As used herein, the term "type 2 autoimmune thyroid disorder" refers to thyroid disorders that include but are not limited to "Grave's disease." As used herein, the term "Grave's disease" refers to symptoms in human patients of hyperactive thyroid function that can be but is not limited to immune system stimulation of thyroid tissue (*e.g.* thyroid hormone receptor stimulating antibodies).

As used herein, the terms "interleukins," "IL," "lymphokines," and "cytokines" refers to molecules that are secreted by cells and tissues including but not limited to white blood cells (*e.g.* leukocytes) and cells in tissues (*e.g.* keratinocytes). As used herein, the abbreviated term "IL" refers to a numbered molecule (*e.g.* IL-2). As used herein, "IL receptors" refers to a receptor for that particular molecule. For example, a receptor for IL-2 is referred to as an IL-2R. IL molecules are received by receptors on the same cell and on other cells. As used herein, the terms "engagement" "stimulation" of interleukin receptors of a cell causes that cell to respond in a variety of ways depending on factors that include but are not limited to its type, differentiation stage, activation stage and cellular context. These responses include but are not limited to proliferation, activation, production of cytokine (*e.g.* increased transcription, increased translation, increase processing, autocrine production, paracrine production and the like), release of cytokines (*e.g.* autocrine, paracrine, portion of cytokine), inhibitory cytokines.

As used herein, the term "contacting" cells with an agent or antibody refers to placing the agent or a antibody in a location that will allow it to touch the cell in order to produce "contacted" cells. The contacting may be accomplished using any suitable method. For example, in one embodiment, contacting is by adding the agent or a antibody to a tube of cells. Contacting may also be accomplished by adding the agent to a culture of the cells. It is not meant to limit how the agent or antibody contacts the cells. In one embodiment, contacting may be accomplished by administration of agent or antibody to an animal *in vivo*.

As used herein, the term "anti-Th1" refers to any agent that reduces the levels and or activities of type 1 cytokines. It is intended that the term be used in its broadest sense, and includes, but is not limited to, agents described herein, for example those which are produced naturally or synthetically.

As used herein, the terms "antigen," "immunogen," "antigenic," "immunogenic," "antigenically active," and "immunologically active" refer to any substance that is capable of inducing a specific humoral or cell-mediated immune response. An immunogen generally contains at least one epitope. Immunogens are exemplified by, but not restricted to molecules which contain a peptide, polysaccharide, nucleic acid sequence, and/or lipid. Complexes of peptides with lipids, polysaccharides, or with nucleic acid sequences are also contemplated, including (without limitation) glycopeptide, lipopeptide, glycolipid, *etc.* These complexes are particularly useful immunogens where smaller molecules with few epitopes do not stimulate a satisfactory immune response by themselves.

As used herein, the term "antigen-presenting cell" and "APC" refers to a term most commonly used when referring to white blood cells that present processed antigenic peptide and MHC class I and/or II molecules to the T-cell receptor on lymphocytes, *(e.g.* macrophages, dendritic cells, B-cells and the like). However, other non-white blood cells can also be referred to as "antigen-presenting cells" since they present peptides within MHC class I and class II to T-cells and the like, *e.g.* as occurs with viral infected cells, cancer cells and the like.

As used herein, the terms "dendritic cell," "DC," and "professional antigen-presenting cells" can evoke an antigen response at least 10X greater in magnitude when compared to

APCs under similar conditions (reviewed in Mellman *et al.* Trends Cell Biol. Jun;8(6):231-7, 1998).

As used herein, the term "cell" refers to a single cell as well as to a population of (*i.e.*, more than one) cells. The population may be a pure population comprising one cell type. alternatively, the population may comprise more than one cell type. In the present invention, there is no limit on the number of cell types that a cell population may comprise.

As used herein, the term "cell culture" refers to any *in vitro* culture of cells. Included within this term are continuous cell lines (*e.g.*, with an immortal phenotype), primary cell cultures, finite cell lines (*e.g.*, non-transformed cells), and any other cell population maintained *in vitro*, including oocytes and embryos.

As used herein, the term "mixed cell culture," refers to a mixture of two or more types of cells. In some embodiments, the cells are cell lines that are not genetically engineered, while in other embodiments the cells are genetically engineered cell lines. In some embodiments the cells contain genetically engineered molecules. The present invention encompasses any combination of cell types suitable for the detection, identification, and/or quantitation of apoptosis in samples, including mixed cell cultures in which all of the cell types used are not genetically engineered, mixtures in which one or more of the cell types are genetically engineered and the remaining cell types are not genetically engineered, and mixtures in which all of the cell types are genetically engineered.

As used herein, the term "primary cell" is a cell which is directly obtained from a tissue (*e.g.* blood) or organ of an animal in the absence of culture. Typically, though not necessarily, a primary cell is capable of undergoing ten or fewer passages *in vitro* before senescence and/or cessation of proliferation. In contrast, a "cultured cell" is a cell which has been maintained and/or propagated *in vitro* for ten or more passages.

As used herein, the term "cultured cells" refer to cells which are capable of a greater number of passages *in vitro* before cessation of proliferation and/or senescence when compared to primary cells from the same source. Cultured cells include "cell lines" and "primary cultured cells."

As used herein, the term "cell line," refers to cells that are cultured *in vitro*, including primary cell lines, finite cell lines, continuous cell lines, and transformed cell lines. but does not require, that the cells be capable of an infinite number of passages in culture. Cell lines may be generated spontaneously or by transformation.

As used herein, the terms "primary cell culture," and "primary culture," refer to cell cultures that have been directly obtained from cells *in vivo*, such as from animal or insect tissue. These cultures may be derived from adults as well as fetal tissue.

As used herein, the terms "monolayer," "monolayer culture," and "monolayer cell culture," refer to cells that have adhered to a substrate and grow as a layer that is one cell in thickness. Monolayers may be grown in any format, including but not limited to flasks, tubes, coverslips (*e.g.*, shell vials), roller bottles, *etc.* Cells may also be grown attached to microcarriers, including but not limited to beads.

As used herein, the term "suspension" and "suspension culture" refers to cells that survive and proliferate without being attached to a substrate. Suspension cultures are typically produced using hematopoietic cells, transformed cell lines, and cells from malignant tumors.

As used herein, the terms "culture media," and "cell culture media," refers to media that are suitable to support the growth of cells *in vitro* (*i.e.*, cell cultures). It is not intended that the term be limited to any particular culture medium. For example, it is intended that the definition encompass outgrowth as well as maintenance media. Indeed, it is intended that the term encompass any culture medium suitable for the growth of the cell cultures of interest.

As used herein the term, the term "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *in vitro* environments exemplified, but are not limited to, test tubes and cell cultures. The term "*in vivo*" refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reactions that occur within a natural environment.

As used herein, the term "proliferation" refers to an increase in cell number.

As used herein, the term "differentiation" refers to the maturation process cells undergo whereby they develop distinctive characteristics, and/or preform specific functions, and/or are less likely to divide.

As used herein, the terms "isolated," "to isolate," "isolation," "purified," "to purify," "purification," and grammatical equivalents thereof as used herein, refer to the reduction in the amount of at least one contaminant (such as protein and/or nucleic acid sequence) from a sample. Thus purification results in an "enrichment," *i.e.*, an increase in the amount of a desirable protein and/or nucleic acid sequence in the sample.

As used herein, the term "amino acid sequence" refers to an amino acid sequence of a naturally occurring or engineered protein molecule. "Amino acid sequence" and like terms, such as "polypeptide," "peptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

As used herein, the term "receptor proteins" and "membrane receptor proteins" refers to membrane spanning proteins that bind a ligand (*e.g.*, a microbial molecule; endotoxin, such as LPS, LTA; dsRNA, and the like).

As used herein, the term "ligand" refers to a molecule that binds to a second molecule. A particular molecule may be referred to as either, or both, a ligand and second molecule. Examples of second molecules include a receptor of the ligand, and an antibody that binds to the ligand.

As is known in the art, "protein phosphorylation" is a common regulatory mechanism used by cells to selectively modify proteins carrying regulatory signals from outside the cell to the cytoplasm and ultimately the nucleus. The proteins that execute these biochemical modifications are a group of enzymes known as protein kinases. They may further be defined by the substrate residue that they target for phosphorylation. One group of protein kinases is the tyrosine kinases (TKs), which selectively phosphorylate a target protein on its tyrosine residues. Some tyrosine kinases are membrane-bound receptors (RTKs), and, upon activation by a ligand, can autophosphorylate as well as modify substrates. The initiation of sequential phosphorylation by ligand stimulation is a paradigm that underlies the action of such effectors as, for example, LPS, LTA, Lethal Toxin (LT), and interferons such as Interferon-.beta. (IFN-.beta.). The receptors for these ligands are tyrosine kinases and provide the interface between the binding of a ligand (hormone, growth factor) to a target cell and the transmission of a signal into the cell by the activation of one or more biochemical pathways. Ligand binding to a receptor tyrosine kinase activates its intrinsic enzymatic activity (See, *e.g.*, Ullrich and

Schlessinger, Cell 61:203-212, 1990). Tyrosine kinases can also be cytoplasmic, non-receptor-type enzymes and act as a downstream component of a signal transduction pathway.

As used herein, the term "protein kinase" refers to a protein that catalyzes the addition of a phosphate group from a nucleoside triphosphate to an amino acid in a protein. Kinases comprise the largest known enzyme superfamily and vary widely in their target proteins. Kinases can be categorized as protein tyrosine kinases (PTKs), which phosphorylate tyrosine residues, and protein serine/threonine kinases (STKs), which phosphorylate serine and/or threonine residues and the like. Some kinases have dual specificity for both serine/threonine and tyrosine residues. Almost all kinases contain a conserved 250-300 amino acid catalytic domain. This domain can be further divided into 11 subdomains. N-terminal subdomains I-IV fold into a two-lobed structure that binds and orients the ATP donor molecule, and subdomain V spans the two lobes. C-terminal subdomains VI-XI bind the protein substrate and transfer the gamma phosphate from ATP to the hydroxyl group of a serine, threonine, or tyrosine residue. Each of the 11 subdomains contains specific catalytic residues or amino acid motifs characteristic of that subdomain. For example, subdomain I contains an 8-amino acid glycine-rich ATP binding consensus motif, subdomain II contains a critical lysine residue that contributes to maximal catalytic activity, and subdomains VI through IX comprise the highly conserved catalytic core. STKs and PTKs also contain distinct sequence motifs in subdomains VI and VIII, which may confer hydroxyamino acid specificity. Some STKs and PTKs possess structural characteristics of both families. In addition, kinases may also be classified by additional amino acid sequences, generally between 5 and 100 residues, which either flank or occur within the kinase domain.

Non-transmembrane PTKs form signaling complexes with the cytosolic domains of plasma membrane receptors. Receptors that signal through non-transmembrane PTKs include cytokine, hormone, and antigen-specific lymphocytic receptors. Many PTKs were first identified as oncogene products in cancer cells in which PTK activation was no longer subject to normal cellular controls. In fact, about one third of the known oncogenes encode PTKs. Furthermore, cellular transformation (oncogenesis) is often accompanied by increased tyrosine phosphorylation activity (See, *e.g.*, Carbonneau, H. and Tonks, Annu. Rev. Cell Biol.

8:463-93, 1992). Regulation of PTK activity may therefore be an important strategy in controlling some types of cancer.

Examples of protein kinases include, but are not limited to, cAMP-dependent protein kinase, protein kinase C, and cyclin-dependent protein kinases (See, *e.g.*, U.S. Pat. Nos. 6,034,228; 6,030,822; 6,030,788; 6,020,306; 6,013,455; 6,013,464; and 6,015,807, all of which are incorporated herein by reference).

As used herein, the term "protein phosphatase" refers to proteins that remove a phosphate group from a protein. Protein phosphatases are generally divided into two groups, receptor-type and non-receptor type (*e.g.* intracellular) proteins. An additional group includes dual specificity phosphatases. Most receptor-type protein tyrosine phosphatases contain two conserved catalytic domains, each of which encompasses a segment of 240 amino acid residues (See *e.g.*, Saito *et al.* Cell Growth and Diff. 2:59, 1991). Receptor protein tyrosine phosphatases can be subclassified further based upon the amino acid sequence diversity of their extracellular domains (See *e.g.*, Krueger *et al.* Proc. Natl. Acad. Sci. USA 89:7417-7421, 1992). Examples of protein phosphatases include, but are not limited to, human protein phosphatase (PROPHO), FIN13, cdc25 tyrosine phosphatase, protein tyrosine phosphatase (PTP) 20, PTP 1D, PTP-D1, PTP .lambda., PTP-S31 (See *e.g.*, U.S. Pat. Nos. 5,853,997; 5,976,853; 5,294,538; 6,004,791; 5,589,375; 5,955,592; 5,958,719; and 5,952,212; all of which are incorporated herein by reference).

As used herein, the term "activating" when in reference to a biochemical response (such as kinase activity) and/or cellular response (such as cell proliferation) refers to increasing the biochemical and/or cellular response.

As used herein, the term "activated" when in reference to a cell, refers to a cell that has undergone a response that alters its physiology and shifts it towards making a biologically response and becoming biologically "active" hence "activated." For example, a monocyte becomes activated to mature into a macrophage. For another example, a macrophage becomes activated upon contact with endotoxin (such as LPS) wherein the activated macrophage can produce an increased level and/or type of molecule associated with activation (*e.g.* iNOS, MMP-12 Metalloelastase and the like). In another example, an immature dendritic cell

becomes activated to mature into a functional dendritic cell. An "activated" cell does not necessarily, although it may, undergo growth or proliferation. Typically, activation of macrophages and DCs, unlike lymphocytes such as T-cells, B-cells and the like, does not stimulate proliferation. Activation can also induce cell death such as in activation-induced cell death (AICD) of T cells. In one embodiment o f the present invention, activation can lead towards apoptotic death.

As used herein, the terms "naturally occurring," "wild-type" and "wt" as used herein when applied to a molecule or composition (such as nucleotide sequence, amino acid sequence, cell, apoptotic blebs, external phosphatidylserine, *etc.*), mean that the molecule or composition can be found in nature and has not been intentionally modified by man. For example, a naturally occurring polypeptide sequence refers to a polypeptide sequence that is present in an organism that can be isolated from a source in nature, wherein the polypeptide sequence has not been intentionally modified by man.

The terms "derived from" and "established from" when made in reference to any cell disclosed herein refer to a cell which has been obtained (*e.g.*, isolated, purified, *etc.*) from the parent cell in issue using any manipulation, such as, without limitation, infection with virus, transfection with DNA sequences, treatment and/or mutagenesis using for example chemicals, radiation, *etc.*, selection (such as by serial culture) of any cell that is contained in cultured parent cells. A derived cell can be selected from a mixed population by virtue of response to a growth factor, cytokine, selected progression of cytokine treatments, adhesiveness, lack of adhesiveness, sorting procedure, and the like.

As used herein, the term "biologically active," refers to a molecules (*e.g.* peptide, nucleic acid sequence, carbohydrate molecule, organic or inorganic molecule, and the like) having structured, regulatory, and/or biochemical functions.

Unless defined otherwise in reference to the level of molecules and/or phenomena, the terms "reduce," "inhibit," "diminish," "suppress," "decrease," and grammatical equivalents when in reference to the level of any molecule (*e.g.*, cytokine protein, nucleic acid sequence, protein sequence, kinase protein, kinase activity, *etc.*), and/or molecular complex (*e.g.* signaling proteins, and the like), phenomenon (*e.g.*, protein-protein interactions, catalytic

activity, apoptosis, cell death, cell survival, cell proliferation, caspase cleavage, receptor dimerization, receptor complex formation, DNA fragmentation, molecule translocation, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) in a first sample relative to a second sample, mean that the quantity of molecule and/or phenomenon in the first sample is lower than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the reduction may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, difficulty in breathing, clarity of vision, nausea, tiredness, *etc.* In another embodiment, the quantity of molecule and/or phenomenon in the first sample is at least 10% lower than, at least 25% lower than, at least 50% lower than, at least 75% lower than, and/or at least 90% lower than the quantity of the same molecule and/or phenomenon in a second sample.

As exemplified herein, in one embodiment, the quantity of substance and/or phenomenon in the first sample is at least 5% lower than the quantity of the same substance and/or phenomenon in a second sample (*e.g.* FIG. 2B, FIG. 6C, and the like). In another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 25% lower than the quantity of the same substance and/or phenomenon in a second sample (*e.g.* FIG. 1B, FIG. 1C, and the like). In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 50% lower than the quantity of the same substance and/or phenomenon in a second sample (*e.g.* FIG. 2B, FIG. 6D, and the like). In a further embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 75% lower than the quantity of the same substance and/or phenomenon in a second sample (*e.g.* FIG. 5B, FIG. 6C, and the like). In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 90% lower than the quantity of the same substance and/or phenomenon in a second sample (*e.g.* FIG. 3C, FIG. 3D and the like). In one embodiment, the reduction may be determined subjectively, for example when comparing mRNA levels (FIG. 2B, FIG. 4A and the like), *etc.*.

As used herein, the term "apoptosis" refers to the process of non-necrotic cell death that takes place in metazoan animal cells following activation of an intrinsic cell suicide

program. Apoptosis is a normal process in the proper development and homeostasis of metazoan animals and usually leads to cell death. Apoptosis is also triggered pathologically by microbial infections resulting in increasing susceptibility to apoptosis and/or outright death. Apoptosis involves sequential characteristic morphological and biochemical changes. One early marker of apoptosis is the flipping of plasma membrane phosphatidylserine, inside to outside, with cellular blebbing called "zeiosis,"of plasma membrane releasing vesicles containing cellular material including RNA and DNA as apoptotic bodies. During apoptosis, there is cell expansion followed by shrinkage through release of apoptotic bodies and lysis of the cell, nuclear collapse and fragmentation of the nuclear chromatin, at certain intranucleosomal sites, due to activation of endogenous nucleases. Apoptotic bodies are typically phagocytosed by other cells, in particular immunocytes such as monocytes, macrophages, immature dendritic cells and the like. One of skill in the art appreciates that reducing the ability to undergo apoptosis results in increased cell survival, without necessarily (although it may include) increasing cell proliferation. Accordingly, as used herein, the terms "reduce apoptosis" and "increase survival" are equivalent. Also, as used herein, the terms "increase apoptosis" and "reduced survival" are equivalent.

Apoptosis may be determined but not limited to, the assays described herein and include methods known in the art. For example, apoptosis may be determined by techniques for detecting DNA fragmentation, (for example any version of the Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End-Labeling TUNEL technique originally developed by Gavrieli *et al.* J Cell Biol. 1992 Nov;119(3):493-501, nuclear staining with nucleic acid dyes such as Hoechst 33342, Acridine Orange and the like, and detecting DNA "ladder" fragmentation patterns associated with apoptosis (*e.g.* DNA gels and the like)). In one embodiment, apoptosis is measured by TUNEL (for example, Park *et al.* Science 297, 2048-51, 2002). In one embodiment apoptosis is measured by observing DNA fragmentation in a ladder pattern (for example, Park *et al.* Science 297, 2048-51, 2002). Apoptosis may be determined by morphological measurements including but not limited to measuring live cells, early apoptotic cells, late apoptotic cells and cell death via apoptosis. For example, the cells' increased display of externally flipped phosphatidylserine, an early indicator of apoptosis, binds external Annexin-V. Thus Annexin-V attached to fluorescent molecules can be used to

stain non permeablized cells and often further combined with vital dyes (*e.g.* trypan blue, propidium Iodide (PI), Ethidium Bromide (EtBr) and the like) allowing fluorescent activated cell sorting (FACS) analysis measuring of live, early apoptotic, late apoptotic and dead cells (Ozawa *et al.* J Exp Med. 1999 Feb 15;189(4):711-8). An example of cell viablilty measurements is demonstrated in Example 2, and described in Example 1. Further, general live v. dead assays may also be employed, for example double staining with EtBr and Calcein AM for live microscopy determinations and FACS. Apoptosis may be determined by the presence of molecular fragments in apoptotic cells not present in live nonapoptotic cells. For example, caspase molecules such as Caspases- 3, 6, 7, and 9 and the like, are cleaved during apoptotic processes, release of cytochrome c, PKR cleavage, and the like. Thus detecting the increased presence of predictable sizes of cleaved caspase subunits in apoptotic cells as compared to nonapoptotic cells indicate that cells are apoptotic. Furthermore, apoptosis may be monitored by changes in protein activity of molecules that decrease or increase cell survival and/or proliferation. For example, protein kinases and nuclear factors increase in activity during apoptosis and serve to either contribute to the apoptotic process or protect against apoptotic damage.

As used herein, the term "cellular response" refers to an increase or decrease of activity by a cell. For example, the "cellular response" may constitute but is not limited to apoptosis, death, DNA fragmentation, blebbing, proliferation, differentiation, adhesion, migration, DNA/RNA synthesis, gene transcription and translation, and/or cytokine secretion or cessation of such processes. A "cellular response" may comprise an increase or decrease of dephosphorylation, phosphorylation, calcium flux, target molecule cleavage, protein-protein interaction, nucleic acid-nucleic acid interaction, and/or protein/nucleic acid interaction and the like. As used herein, the term "target molecule cleavage" refers to the splitting of a molecule (for example in the process of apoptosis, cleavage of procaspases into fragments, cleavage of DNA into predicable sized fragments and the like). As used herein, the term "interaction" refers to the reciprocal action or influence of two or more molecules on each other.

As used herein, the term "phosphorylation" refers to the addition of phosphate groups. Protein phosphorylation is catalyzed by protein kinases which attach phosphate groups to

hydroxyls of Ser, Thr and/or Tyr side chains. As used herein, the term "dephosphorylation" refers to the removal of a phosphate group. Protein dephosphorylation is catalyzed by protein phosphatases which remove phosphate groups from the side chains of Ser, Thr, and/or Tyr.

As used herein, the term "transgenic" when used in reference to a cell which contains a transgene, or whose genome has been altered by the introduction of a transgene. The term "transgenic" when used in reference to a tissue or to a plant refers to a tissue or plant, respectively, which comprises one or more cells that contain a transgene, or whose genome has been altered by the introduction of a transgene. Transgenic cells, tissues and plants may be produced by several methods including the introduction of a "transgene" comprising nucleic acid (usually DNA) into a target cell or integration of the transgene into a chromosome of a target cell by way of human intervention, such as by the methods described herein.

As used herein, the term "transgene" as used herein refers to any nucleic acid sequence which is introduced into the cell by experimental manipulations. A transgene may be an "endogenous DNA sequence" or a "heterologous DNA sequence" (*i.e.*, "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence which is naturally found in the cell into which it is introduced so long as it does not contain some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, *etc.*) relative to the naturally-occurring sequence. The term "heterologous DNA sequence" refers to a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Heterologous DNA also includes an endogenous DNA sequence which contains some modification. Generally, although not necessarily, heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is expressed. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, selectable marker proteins (*e.g.*, proteins which confer drug resistance), *etc.*

As used herein, the terms "agent," "test agent," "molecule," "test molecule," "compound," and "test compound" as used interchangeably herein, refer to any type of molecule (for example, a peptide, nucleic acid, carbohydrate, lipid, organic molecule, and inorganic molecule, *etc.*) any combination molecule for example glycolipid, *etc.*) obtained from any source (for example, plant, animal, protist, and environmental source, *etc.*), or prepared by any method (for example, purification of naturally occurring molecules, chemical synthesis, and genetic engineering methods, *etc.*). Test agents are exemplified by, but not limited to individual and combinations of antibodies, nucleic acid sequences, and other agents as further described below.

In one embodiment, the term "test agent" refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function. Test agents comprise both known and potential therapeutic agents. A test agent can be determined to be therapeutic by screening using the screening methods of the present invention. A "known therapeutic agent" refers to a therapeutic agent that has been shown (*e.g.*, through animal trials or prior experience with administration to humans) to be effective in such treatment or prevention. In other words, a known therapeutic agent is not limited to an agent efficacious in the treatment of disease (*e.g.*, cancer). Agents are exemplified by, but not limited to, antibodies, nucleic acid sequences such as ribozyme sequences, and other agents as further described herein.

The test agents identified by and/or used in the invention's methods include any type of molecule (for example, a peptide, nucleic acid, carbohydrate, lipid, organic, and inorganic molecule, *etc.*) obtained from any source (for example, plant, animal, and environmental source, *etc.*), or prepared by any method (for example, purification of naturally occurring molecules, chemical synthesis, and genetic engineering methods, *etc.*).

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth as used herein, are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters herein are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the

very least, and without limiting the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters describing the broad scope of the invention are approximations, the numerical values in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains standard deviations that necessarily result from the errors found in the numerical value's testing measurements.

The term "not" when preceding, and made in reference to, any particularly named molecule (*e.g.*, nucleic acid sequence, protein sequence, apoptotic blebs, external phosphatidylserine, *etc.*), and/or phenomenon (*e.g.*, apoptosis, cell death, cell survival, cell proliferation, caspase cleavage, receptor dimerization, receptor complex formation, DNA fragmentation, molecule translocation, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) means that primarily the particularly named molecule or phenomenon is excluded.

The term "altering" and grammatical equivalents as used herein in reference to the level of any molecule (*e.g.*, nucleic acid sequence, protein sequence, apoptotic blebs, external phosphatidylserine, *etc.*), and/or phenomenon (*e.g.*, apoptosis, cell death, cell survival, cell proliferation, caspase cleavage, receptor dimerization, receptor complex formation, DNA fragmentation, molecule translocation, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) refers to an increase and/or decrease in the quantity of the molecule and/or phenomenon, regardless of whether the quantity is determined objectively, and/or subjectively.

Unless defined otherwise in reference to the level of molecules and/or phenomena, the terms "increase," "elevate," "raise," and grammatical equivalents when in reference to the level of any molecule (*e.g.*, nucleic acid sequence, protein sequence, apoptotic blebs, external phosphatidylserine, *etc.*), and/or phenomenon (*e.g.*, apoptosis, cell death, cell survival, cell proliferation, caspase cleavage, receptor dimerization, receptor complex formation, DNA fragmentation, molecule translocation, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) in a first sample

relative to a second sample, mean that the quantity of the molecule and/or phenomenon in the first sample is higher than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the increase may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, difficulty in breathing, clarity of vision, nausea, tiredness, *etc.* In another embodiment, the quantity of the molecule and/or phenomenon in the first sample is at least 10% greater than, at least 25% greater than, at least 50% greater than, at least 75% greater than, and/or at least 90% greater than the quantity of the same molecule and/or phenomenon in a second sample.

Reference herein to any specifically named protein (such as mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch *etc.*) refers to any and all equivalent fragments, fusion proteins, and variants of the specifically named protein, having at least one of the biological activities (such as those disclosed herein and/or known in the art) of the specifically named protein, wherein the biological activity is detectable by any method.

The term "fragment" when in reference to a protein (such as mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch *etc.*) refers to a portion of that protein that may range in size from four (4) contiguous amino acid residues to the entire amino acid sequence minus one amino acid residue. Thus, a polypeptide sequence comprising "at least a portion of an amino acid sequence" comprises from four (4) contiguous amino acid residues of the amino acid sequence to the entire amino acid sequence.

The term "fusion protein" refers to two or more polypeptides that are operably linked. The term "operably linked" when in reference to the relationship between nucleic acid sequences and/or amino acid sequences refers to linking the sequences such that they perform their intended function. For example, operably linking a promoter sequence to a nucleotide sequence of interest refers to linking the promoter sequence and the nucleotide sequence of interest in a manner such that the promoter sequence is capable of directing the transcription of the nucleotide sequence of interest and/or the synthesis of a polypeptide encoded by the nucleotide sequence of interest. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "variant" of a protein (such as mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch, *etc.*) as used herein is defined as an amino acid sequence which differs by insertion, deletion, and/or conservative substitution of one or more amino acids from the protein of which it is a variant. The term "conservative substitution" of an amino acid refers to the replacement of that amino acid with another amino acid which has a similar hydrophobicity, polarity, and/or structure. For example, the following aliphatic amino acids with neutral side chains may be conservatively substituted one for the other: glycine, alanine, valine, leucine, isoleucine, serine, and threonine. Aromatic amino acids with neutral side chains which may be conservatively substituted one for the other include phenylalanine, tyrosine, and tryptophan. Cysteine and methionine are sulphur-containing amino acids which may be conservatively substituted one for the other. Also, asparagine may be conservatively substituted for glutamine, and *vice versa*, since both amino acids are amides of dicarboxylic amino acids. In addition, aspartic acid (aspartate) may be conservatively substituted for glutamic acid (glutamate) as both are acidic, charged (hydrophilic) amino acids. Also, lysine, arginine, and histidine my be conservatively substituted one for the other since each is a basic, charged (hydrophilic) amino acid. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological and/or immunological activity may be found using computer programs well known in the art, for example, DNAStar™ software. In one embodiment, the sequence of the variant has at least 95% identity, at least 90% identity, at least 85% identity, at least 80% identity, at least 75% identity, at least 70% identity, and/or at least 65% identity with the sequence of the protein in issue.

Reference herein to any specifically named nucleotide sequence (such as a sequence encoding mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch, *etc.*) includes within its scope any and all equivalent fragments, homologs, and sequences that hybridize under highly stringent and/or medium stringent conditions to the specifically named nucleotide sequence, and that have at least one of the biological activities (such as those disclosed herein and/or known in the art) of the specifically named nucleotide sequence, wherein the biological activity is detectable by any method.

The "fragment" or "portion" may range in size from an exemplary 5, 10, 20, 50, or 100 contiguous nucleotide residues to the entire nucleic acid sequence minus one nucleic acid residue. Thus, a nucleic acid sequence comprising "at least a portion of" a nucleotide sequence (such as sequences encoding mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch, *etc.*) comprises from five (5) contiguous nucleotide residues of the nucleotide sequence to the entire nucleotide sequence.

The term "homolog" of a specifically named nucleotide sequence refers to an oligonucleotide sequence which exhibits greater than 50% identity to the specifically named nucleotide sequence (such as a sequence encoding mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch, *etc*). Alternatively, or in addition, a homolog of a specifically named nucleotide sequence is defined as an oligonucleotide sequence which has at least 95% identity, at least 90% identity, at least 85% identity, at least 80% identity, at least 75% identity, at least 70% identity, and/or at least 65% identity to nucleotide sequence in issue.

With respect to sequences that hybridize under stringent conditions to the specifically named nucleotide sequence (such as a sequence encoding mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch, *etc.*), high stringency conditions comprise conditions equivalent to binding or hybridization at 68°C in a solution containing 5X SSPE, 1% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution containing 0.1X SSPE, and 0.1% SDS at 68°C. "Medium stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH_2PO_4-H_2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution comprising 1.0X SSPE, 1.0% SDS at 42°C.

The term "equivalent" when made in reference to a hybridization condition as it relates to a hybridization condition of interest means that the hybridization condition and the hybridization condition of interest result in hybridization of nucleic acid sequences which have the same range of percent (%) homology. For example, if a hybridization condition of interest results in hybridization of a first nucleic acid sequence with other nucleic acid

sequences that have from 85% to 95% homology to the first nucleic acid sequence, then another hybridization condition is said to be equivalent to the hybridization condition of interest if this other hybridization condition also results in hybridization of the first nucleic acid sequence with the other nucleic acid sequences that have from 85% to 95% homology to the first nucleic acid sequence.

As will be understood by those of skill in the art, it may be advantageous to produce a nucleotide sequence encoding a protein of interest, wherein the nucleotide sequence possesses non-naturally occurring codons. Therefore, in some embodiments, codons preferred by a particular prokaryotic or eukaryotic host (Murray *et al.*, Nucl. Acids Res., 17 (1989)) are selected, for example, to increase the rate of expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

A "composition" comprising a particular polynucleotide sequence (such as a sequence encoding mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch, *etc.*) and/or comprising a particular protein sequence (such as mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch, *etc.*) as used herein refers broadly to any composition containing the recited polynucleotide sequence (and/or its equivalent fragments, homologs, and sequences that hybridize under highly stringent and/or medium stringent conditions to the specifically named nucleotide sequence) and/or the recited protein sequence (and/or its equivalent fragments, fusion proteins, and variants), respectively. The composition may comprise an aqueous solution containing, for example, salts (*e.g.*, NaCl), detergents (*e.g.*, SDS), and other components (*e.g.*, Denhardt's solution, dry milk, salmon sperm DNA, *etc.*).

The terms nucleotide sequence "comprising a particular nucleic acid sequence" and protein "comprising a particular amino acid sequence" and equivalents of these terms, refer to any nucleotide sequence of interest (such as a sequence encoding mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch, *etc.*) and to any protein of interest (such as mitogen-activated protein kinase/ERK kinase kinase 1, C-Jun N-terminal kinase 1, itch, *etc.*), respectively, that contain the particularly named nucleic acid sequence

(and/or its equivalent fragments, homologs, and sequences that hybridize under highly stringent and/or medium stringent conditions to the specifically named nucleotide sequence) and the particularly named amino acid sequence (and/or its equivalent fragments, fusion proteins, and variants), respectively. The invention does not limit the source (*e.g.*, cell type, tissue, animal, *etc.*), nature (*e.g.*, synthetic, recombinant, purified from cell extract, *etc.*), and/or sequence of the nucleotide sequence of interest and/or protein of interest. In one embodiment, the nucleotide sequence of interest and protein of interest include coding sequences of structural genes (*e.g.*, probe genes, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*).

As used herein, the terms "cascade pathway," signaling pathway," "signal transduction pathway," "MAP kinase pathway" refers to an intracellular signaling system involving a network of proteins that include but are not limited to the MAP kinase cascades. An intracellular signaling system includes but is not limited to a response to extracellular stimuli that "triggers" a cascade by activating the first members (upstream activators) of a cascade. An general example of an upstream activator includes but is not limited to map kinase kinase kinases (MAPKKKs), these in turn induce phosphorylation of mitogen-activated protein kinase kinases (MAPKK) which in turn phosphorylate the mitogen-activated protein kinase; (MAPKs). The MAPKs then act on various downstream targets to affect gene expression. Examples of mammalian MAP kinase pathways including ERK (extracellular signal-regulated kinase) pathway, SAPK/JNK (stress-activated protein kinase/c-jun kinase) pathway, and p38 kinase pathway. It is not meant that these pathways are separate, in some embodiments, there is sharing of components among the pathways depending on many factors including but not limited to which stimulus originates activation of the cascade, the strength of stimulus, presence and strength of co-stimulatory signals, differentiation stage of the cell, activation stage of the cell, *etc.*

DESCRIPTION OF THE INVENTION

The present invention relates to T helper 2 (Th2) cytokine production, and in particular, biasing the cytokine expression profile towards Th2 cytokine production through

mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1), the screening of agents that increase Th2 cytokine production, and the treatment of T helper 1 (Th1) biased autoimmune diseases *in vivo*. In one embodiment, the present invention relates to agents including but not limited to reducing the activity of MEKK1, leading to increased levels of Th2 cytokine production.

Engagement of T cell receptor (TCR) along with co-stimulatory receptors, such as CD28, results in activation of several signaling pathways that stimulate the activity of various transcription factors involved in production of T cell cytokines (Crabtree *et al.* (1994) Annu Rev Biochem 63, 1045-1083; Weiss *et al.* (1994) Cell 76, 263-274). As used herein, the term "engagement" refers to when a receptor is triggered in such a way as to induce a biochemical response (*e.g.* activation of a signaling pathway and the like as demonstrated herein using anti-CD3 antibodies). As used herein, the term "T cell receptor," "T-cell antigen receptor," and "TCR" refers to T-cell antigen receptor typically consisting of either an a/b dimer or a g/d dimer associated with the CD3 molecular complex, *in vivo*. As used herein, the term "CD3 molecular complex" refers to the association of molecules that result in cellular biochemical responses.

These transcription factors include NF-AT and NF-kB family members, as well as members of the AP-1 family (Crabtree *et al.* (1994) Annu Rev Biochem 63, 1045-1083; Nolan (1994) Cell 77, 795-798; Rao *et al.* (1997) Annu Rev Immunol 15, 707-747; Weiss *et al.* (1994) Cell 76, 263-274). In addition to cytokine gene induction, it was demonstrated that the intensity and duration of the signal generated by engagement of TCR and co-stimulatory receptors can modulate the differentiation of naïve T helper (Th) cells into the Th1 and Th2 effector subsets (Boyton *et al.* (2002) Trends Immunol 23, 526-529; Constant *et al.* (1995) J Exp Med 182, 1591-1596; Itoh *et al.* (1997) J Exp Med 186, 757-766; Kuchroo *et al.* (1995) Cell 80, 707-718; Lanzavecchia *et al.* (2000) Science 290, 92-97). While the mechanisms involved activation of T cell cytokine genes are relatively well understood (Murphy *et al.* (2000) Annu Rev Immunol 18, 451-494), the biochemical mechanisms by which TCR-generated signals can modulate the spectrum of cytokine gene expression are unknown. Nonetheless, it was observed that Th1 and Th2 cells exhibit fundamental differences in Ca2+

signaling (Gajewski *et al.* (1990) J Immunol 144, 4110-4120; Sloan-Lancaster *et al.* (1997) J Immunol 159, 1160-1168).

Some of the transcription factors required for Th cell polarization and differentiation were identified (Murphy *et al.* (2000) Annu Rev Immunol 18, 451-494). These include T-bet which is required for commitment to the Th1 phenotype (Szabo *et al.* (2000) Cell 100, 655-669) and GATA3 (Zheng *et al.* (1997) Cell 89, 587-596), c-Maf (Ho *et al.* (1996) Cell 85, 973-983), and JunB (Hartenstein *et al.* (2002) Embo J 21, 6321-6329; Li *et al.* (1999) Embo J 18, 420-432) for the Th2 phenotype. JunB is a member of the AP-1 family of transcription factors, which also includes c-Jun and JunD, as well as c-Fos, FosB, Fra1 and Fra2 and several other proteins (Angel *et al.* (1991) Biochim Biophys Acta 1072, 129-157). AP-1 activity is subject to complex regulation, in which members of the mitogen activated protein kinase (MAPK) family play an important role both by regulating the expression of Jun and Fos genes, as well as by modulating the transcriptional activity of their protein products through direct phosphorylation (Karin (1995) J Biol Chem 270, 16483-16486). Amongst the key regulators of AP-1 activity are the Jun N-terminal kinases (JNKs), which in addition to the Jun proteins (Kallunki *et al.* (1996) Cell 87, 929-939), also phosphorylate other transcription factors (Minden *et al.* (1997) Biochim Biophys Acta 1333, F85-104). Importantly, in T cells the JNKs are primary members of the MAPK family whose activation in response to TCR engagement is strongly potentiated by occupancy of the co-stimulatory receptor CD28 (Su *et al.* (1994) Cell 77, 727-736). These findings suggested that JNK may be involved in the interpretation of co-stimulatory signals or the intensity of T cell activation. TCR-mediated JNK activation depends on Ca2+ signaling (Werlen *et al.* (1998) Embo J 17, 3101-3111).

As used herein, "co-stimulatory signals," "co-stimulatory molecules," "co-stimulatory stimulation," "stimulatory signal," and "co-stimulation" refers to interactions between cells of the immune system wherein a costimulatory "second messenger" molecule controls the cell-to-cell transmission of a signal that initiates an immune response. For example, CD28 is a receptor refered to as a co-stimulatory molcule in that triggering of CD28 in addition to

CD3 enhances the CD3 response. It is not meant that co-stimulation be limited to receptors in that co-stimulation can be accomplished by the use of antibodies (*e.g.* Example 2).

In addition to direct binding to several cytokine gene promoters, such as that of IL-4 (Li *et al.* (1999) Embo J 18, 420-432; Rooney *et al.* (1995) Immunity 2, 473-483), AP-1 proteins are required for cooperative binding of NF-AT proteins to low affinity sites (Rao *et al.* (1997) Annu Rev Immunol 15, 707-747; Rooney *et al.* (1995) Immunity 2, 473-483). While all three Jun proteins are expressed in T cells, it was found that differentiation towards the Th2 phenotype is accompanied by up-regulation of JunB, whose ectopic expression can polarize naïve Th cells towards the Th2 phenotype (Li *et al.* (1999) Embo J 18, 420-432). Gene disruption experiments confirmed the critical role of JunB in expression of Th2 cytokines (Hartenstein *et al.* (2002) Embo J 21, 6321-6329), which include IL-4, IL-5, IL-10 and IL-13 (Murphy *et al.* (2000) Annu Rev Immunol 18, 451-494; Paul *et al.* (1994) Cell 76, 241-251). JunB can directly activate the IL-4 promoter in cooperation with c-Maf and this activity is strongly potentiated by JNK-mediated JunB phosphorylation (Li *et al.* (1999) Embo J 18, 420-432). Despite the ability of JNK to enhance JunB (and c-Jun) transcriptional activity, the loss of both JNK1 and JNK2 expression strongly enhances, rather than attenuates, the generation of Th2 effector cells (Dong *et al.* (2000) Nature 405, 91-94). These results suggest that the JNKs may have a complex and rather enigmatic role in regulation of JunB or other transcription factors required for expression of Th2 cytokines.

As used herein, the terms "JNK1," "c-Jun N-terminal kinase 1," "Mitogen-activated protein kinase 8," "Stress-activated protein kinase JNK1," and "JNK-46" refers to the protein encoded by genes including but not limited to "JNK1" or "MAPK8" or "PRKM8."

As used herein, the terms "JNK2," "c-Jun N-terminal kinase 2," "Mitogen-activated protein kinase 9," "Stress-activated protein kinase JNK2," and "JNK-55" refers to the protein encoded by genes including but not limited to "JNK2" or "MAPK9" or "PRKM9."

Another protein that is involved in the regulation of Jun protein activity or expression in T cells is the E3 ubiquitin ligase Itch (Fang *et al.* (2002) Nat Immunol 3, 281-287).

As used herein, the terms "Itchy E3 ubiquitin protein ligase," "itch E3 ubiquitin protein ligase," "E3 ubiquitin protein ligase itch," "Itchy homolog E3 ubiquitin protein

ligase," "Itch," itchy," "Atrophin-1-interacting protein 4,""AIP4,""NFE2-associated polypeptide 1," and "NAPP1" refers to mouse and human proteins whose gene names include but are not limited to "ITCH."

Itchy mutant mice, which express an inactive form of Itch, exhibit increased expression of Th2 cytokines as well as elevated levels of c-Jun and JunB in their T cells (Fang *et al.* (2002) Nat Immunol 3, 281-287). Itch is a member of the HECT domain group of E3 ubiquitin-protein ligases (Qiu *et al.* (2000) J Biol Chem 275, 35734-35737). These proteins recognize their substrates via a WW domain, which unlike the F boxes used by members of the SCF group of ubiquitin-protein ligases, do not recognize phospho-epitopes (Ciechanover *et al.* (2000) Bioessays 22, 442-451; Joazeiro *et al.* (2000) Science 289, 2061-2062). Yet, Itch was shown to interact with both JunB and c-Jun and stimulate their polyubiquitination in an *in vitro* system (Fang *et al.* (2002) Nat Immunol 3, 281-287).

To further understand the role of the JNK pathway in regulation of AP-1 and Jun activity in T cells, we analyzed the role of the MAPK kinase (MAP2K) kinase (MAP3K) MEKK1 in TCR-mediated JNK activation and gene expression.

As used herein, the terms "MEKK1," "Mitogen-activated protein kinase kinase kinase 1," "MAP3K," "MAPK/ERK kinase kinase 1," "MEK kinase 1," and "mitogen-activated protein kinase/ERK kinase kinase 1" refers to mouse and human proteins whose gene names include but are not limited to "MEKK 1" "MAP3K1" or "MAPKKK1" or "MEKK."

As used herein, the terms "MEKK4," Mitogen-activated protein kinase kinase kinase 4," " MAPK/ERK kinase kinase 4," "MEK kinase 4," "MEKK 4" refers to mouse and human proteins whose gene names include but are not limited to "MAP3K4" or "MAPKKK4" or "MEKK4" or "MTK1" or "KIAA0213."

As used herein, the terms "MEKK7," "Dual specificity mitogen-activated protein kinase kinase 7," "MAP kinase kinase 7," "MAPKK 7," "MAPK/ERK kinase 7," "JNK activating kinase 2," "c-Jun N-terminal kinase kinase 2," "JNK kinase 2," "JNKK 2," "MKK7" refers to mouse and human proteins whose gene names include but are not limited to "MAP2K7" or "MKK7" or "MAP2K7" or "PRKMK7" or "JNKK2" or "MKK7."

MEKK1 is one of the most potent activators of the JNK cascade identified (Minden *et al.* (1997) Biochim Biophys Acta 1333, F85-104). Here we show that MEKK1 is the major activator of the JNK cascade in response to TCR and CD28 co-ligation. Interestingly, disruption of MEKK1 catalytic activity or JNK inhibition results in marked overexpression of Th2 cytokines while having no effect on Th1 cytokines. Furthermore, like the inactivation of Itch, the loss of MEKK1 catalytic activity results in stabilization of c-Jun and JunB. We show that MEKK1 physically interacts with Itch and can regulate its stability and enhance the polyubiquitination of JunB. Based on these results we suggest that TCR and CD28 co-ligation results in activation of the MEKK1 to JNK cascade, which has two opposing effects on JunB and c-Jun. While enhancing their transcriptional activity through direct phosphorylation, it also leads to their increased turnover and eventual degradation. The latter effect could be the biochemical mechanism through which output from the TCR and co-stimulatory receptors modulates Th cell differentiation.

RESULTS

TCR hyperresponsiveness in Mekk1^{KD} mutant T cells

Although the role of JNK1 and JNK2, as well as the upstream kinase MKK7, in both thymocyte and peripheral T cell activation has been examined, some of the results were rather controversial (Dong *et al.* (2000) Nature 405, 91-94; Sabapathy *et al.* (2001) J Exp Med 193, 317-328; Sasaki *et al.* (2001) J Exp Med 194, 757-768) and as discussed above inconsistent with results obtained for the JNK substrate JunB (Hartenstein *et al.* (2002) Embo J 21, 6321-6329; Li *et al.* (1999) Embo J 18, 420-432). We were interested in understanding whether MEKK1 is the MAP3K responsible for JNK activation in response to TCR engagement and if so examine its function in T cell biology. The loss of MEKK1 catalytic activity (Xia *et al.* (2000) Proc Natl Acad Sci U S A 97, 5243-5248) had no effect on thymocyte numbers and differentiation into the CD4 and CD8 double and single positive classes (unpublished results). However, MEKK1 kinase-deficient (Mekk1^{KD}) thymocytes were hyperresponsive to TCR engagement with anti-CD3 antibody and this hyperresponsiveness was strongly augmented by ligation of the co-stimulatory CD28 receptor (Figure 1A).

Proliferation in response to PMA and ionomycin, stimuli that bypass the TCR and CD28, was not affected (data not shown). The increased proliferative response of Mekk1^{KD} thymocytes was not due to decreased activation-induced death (Figure 1B). In fact, activation-induced death was slightly elevated in mutant cells. In addition, the expression levels of several cell death regulators were unchanged in mutant thymocytes (data not shown). Similar, but not as pronounced, hyperresponsiveness was observed in peripheral Mekk1^{KD} T cells (Figure 1C), whereas the rate of cell death was comparable between WT and Mekk1^{KD} splenic T cells (data not shown).

Decreased JNK activation in Mekk1^{KD} mutant thymocytes underlies their hyperresponsiveness

We next examined the effect of the Mekk1^{KD} mutation on activation of JNK and other MAPKs in response to TCR and CD28 engagement. Loss of MEKK1 catalytic activity resulted in a considerable decrease in JNK activation following stimulation of thymocytes with anti-CD3 + anti-CD28 (Figure 2A). By contrast, ERK activation was not affected (Figure 2B). p38 MAPK was not activated by these stimuli in either WT or Mekk1^{KD} thymocytes (data not shown). To determine whether loss of JNK activity causes TCR hyperresponsiveness, we used the small molecule JNK inhibitor SP600125 (Bennett *et al.*, 2001). Stimulation of WT thymocytes with different concentrations of anti-CD3 + anti-CD28 in the presence of increasing amounts of SP600125, which inhibited JNK activity (see below), resulted in at least 2-fold increase in [3H] thymidine incorporation (Figure 2C). To confirm that the effect of MEKK1 on thymocyte proliferation is mediated through JNK, we bred Jnk1$^{-/-}$ mice with Mekk1^{KD} mice. Interestingly, Mekk1$^{+/KD}$Jnk1$^{+/-}$ mice showed open eyelids at birth, the same phenotype exhibited by Mekk1$^{KD/KD}$ mice, while both Mekk1$^{+/KD}$ and Jnk1$^{-/-}$ mice were born with closed eyes (data not shown). Importantly, Mekk1$^{+/-}$Jnk1$^{+/-}$ thymocytes exhibited a pronounced hyperresponsiveness to TCR and CD28 engagement in comparison to either Jnk1$^{+/-}$ (Figure 2D) or Mekk1+/KD (data not shown) thymocytes.

Increased Th2 cytokine production by Mekk1^{KD} thymocytes

To determine the mechanism by which reduced JNK and MEKK1 activity leads to thymocyte and T cell hyperresponsiveness, we compared the pattern of TCR and downstream effector protein tyrosine phosphorylation between WT and mutant cells and found negligible obvious differences (data not shown). We also compared the levels of various cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors, including p16, between WT and mutant thymocytes and found negligible major differences in their expression (data not shown). Next we considered the role of cytokine gene expression in the hyperresponsive phenotype of Mekk1KD T cells, as cytokines play crucial roles in regulating T cell activation and differentiation (Murphy *et al.* (2000) Annu Rev Immunol 18, 451-494; Paul *et al.* (1994) Cell 76, 241-251; Smith *et al.* (1979) Ann N Y Acad Sci 332, 423-432). Indeed, Mekk1KD thymocytes were found to express elevated levels of IL-4 and IL-13 mRNAs but close to normal levels of interferon-gamma (IFN-g) mRNA after stimulation with anti-CD3 + anti-CD28 (Figure 3A). Unstimulated thymocytes expressed primarily background levels of these mRNAs (data not shown). In addition to ribonuclease (RNase) protection analysis, we used a more sensitive real-time PCR assay to quantitate the levels of these and other mRNAs in WT and Mekk1KD thymocytes after 48 hrs stimulation with anti-CD3 + anti-CD28. While expression of IL-2 or IFN-g mRNAs was not affected, Mekk1^{KD} cells expressed up to 5-fold more IL-4, IL-5, IL-10 and IL-13 mRNAs (Figure 3B). Upregulation of IL-4 mRNA was also observed by microarray analysis (data not shown). The cytokine mRNAs that are overexpressed in Mekk1^{KD} thymocytes are characteristic of the Th2 effector cell type (Murphy *et al.* (2000) Annu Rev Immunol 18, 451-494; Paul *et al.* (1994) Cell 76, 241-251). Indeed, when induced to differentiate, naïve Mekk1^{KD} Th cells exhibited a marked bias towards the Th2 type (T. Naumaner, unpublished data).

To determine whether increased IL-4 production was responsible for the hyperproliferation of Mekk1^{KD} thymocytes, we examined the effect of a neutralizing anti-IL-4 antibody. Incubation with anti-IL-4, but not control anti-IgG, markedly reduced the proliferation of Mekk1^{KD} thymocytes elicited by anti-CD3 in the absence or presence of anti-CD28 (Figure 3C and data not shown). In addition, addition of exogenous IL-4 to WT thymocytes strongly potentiated their proliferative response to the same simuli (Figure 3D).

Elevated c-Jun and JunB expression in Mekk1^{KD} thymocytes

The molecular regulation of IL-4, IL-5, and IL-13 gene expression was recently reviewed (Murphy *et al.* (2000) Annu Rev Immunol 18, 451-494). Multiple transcription factors, including NF-AT, GATA-3, c-Maf, JunB, are involved in IL-4 gene induction (Murphy *et al.*, 2000; Paul and Seder, 1994). JunB (Li *et al.*, 1999), as well as its relative c-Jun (Hibi *et al.* (1993) Genes Dev 7, 2135-2148; Kallunki *et al.* (1996) Cell 87, 929-939), are targets for JNK-mediated phosphorylation. Curiously, we found that both c-Jun and JunB were elevated in activated Mekk1^{KD} thymocytes, while expression of JunD, another family member, was unchanged (Figure 4A). Mekk1^{KD} cells, also expressed normal levels of GATA-3, c-Maf, NF-ATc1 and NF-ATc2. Surprisingly, despite the change in protein levels, the levels of c-Jun and JunB mRNAs were not different between WT and mutant thymocytes (Figure 4B).

To confirm that JunB, and possibly c-Jun, functions as a transcriptional regulators of the IL-4 gene, we performed chromatin immunoprecipitation (ChIP) experiments. Recruitment of both c-Jun and JunB to the proximal IL-4 promoter region was detected in both WT and Mekk1^{KD} thymocytes activated with anti-CD3 + anti-CD28 (Figure 4C). Neither protein was recruited to the IL-4 coding region. Control experiments with anti-p65(RelA) antibodies did not result in precipitation of the proximal IL-4 promoter region (data not shown), consistent with the absence of NF-kB binding sites (Murphy *et al.* (2000) Annu Rev Immunol 18, 451-494). In multiple repeats of this experiment we observed that the IL-4 promoter signal was modestly, but reproductively, more intense when the ChIP was performed on mutant cells.

MEKK1 promotes ubiquitin-dependent degradation of c-Jun and JunB

A possible mechanism through which MEKK1 and JNK can modulate c-Jun and JunB levels is by promoting their turnover. To examine this point, we conducted pulse-chase experiments. In WT thymocytes, newly synthesized c-Jun and JunB proteins were degraded with half-lives (t1/2) of 59 and 78 min, respectively (Figure 5A,B). Both proteins were significantly more stable in Mekk1^{KD} thymocytes, where their t1/2 was extended to 109 and

280 min, respectively. By contrast, the turnover of RelA(p65) was unaltered between WT and Mekk1^{KD} cells.

Degradation of many short-lived transcription factors is controlled by ubiquitin-dependent proteolysis (Hershko *et al.* (1998) Annu Rev Biochem 67, 425-479). We examined whether MEKK1 is involved in c-Jun or JunB ubiquitination. Due to difficulties in detecting endogenously ubiquitinated proteins, we used transiently transfected cells. 293T cells were transfected with plasmids encoding Myc-tagged ubiquitin, hemagglutinin (HA)-tagged c-Jun or JunB, and either WT or kinase domain-truncated MEKK1. Cell lysates were immunoprecipitated with anti-HA, and ubiquitination of c-Jun and JunB was monitored by immunoblotting with anti-Myc. Ectopic expression of WT MEKK1 promoted Ub conjugation to both c-Jun and JunB (Figure 5C, data not shown for c-Jun). By contrast, overexpression of the MEKK1 mutant resulted in weaker Jun polyubiquitination. These data suggest that MEKK1 promotes c-Jun and JunB turnover by enhancing the extent of their ubiquitination.

The MEKK1/JNK cascade regulates Itch turnover and expression, as well as Jun ubiquitination and cytokine production

An important role in protein ubiquitination is played by the E3 ubiquitin protein ligases, which are responsible for substrate recognition (Hershko *et al.* (1998) Annu Rev Biochem 67, 425-479). Elevated expression of c-Jun and JunB and increased Th2 cytokine production were recently described in T cells from Itchy mutant mice, that express an inactive form of the Itch E3 ubiquitin protein ligase. These similarities raised the possibility that MEKK1-directed JunB (or c-Jun) turnover is Itch-dependent and that the MEKK1/JNK cascade controls Itch activity or expression. We found that Mekk1^{KD} thymocytes expressed lower levels of Itch before and after activation (Figure 6A and data not shown). Although decreased Itch expression was also observed in Mekk1^{KD} splenic T cells and B cells, Itch was expressed at normal levels in the heart and liver of mutant mice (data not shown). Importantly, Itch and MEKK1 exhibited very efficient and nearly quantitative interaction in thymocytes and splenic T cells (Figure 6B and data not shown).

Despite reduced Itch protein, the level of Itch mRNA was identical between WT and Mekk1^{KD} lymphocytes (data not shown). To investigate whether MEKK1 controls Itch protein turnover, we conducted pulse-chase experiments. In Mekk1^{KD} cells, newly synthesized Itch was degraded with a t1/2 of 77 min, whereas in WT cells its t1/2 was 136 min (Figure 6C). Thus the absence of MEKK1 kinase activity results in destabilization of Itch, while it stabilizes c-Jun and JunB.

To test whether this effect of MEKK1 is mediated through JNK, we used the JNK inhibitor. Incubation of activated WT thymocytes with SP600125 resulted in a 4-fold increase in c-Jun and JunB expression and a 2-fold decrease in Itch expression after 24 hrs (Figure 6D). Negligible such effects were observed upon incubation with the p38 inhibitor SB202190 (data not shown). The effect of SP600125 on Jun and Itch expression correlated with its effect of JNK activation (Figure 6E). We also found that treatment with the JNK inhibitor reduced the extent of c-Jun and JunB polyubiquitination in transfected cells (Figure 6F). In addition to the effects on Jun and Itch expression, treatment of activated thymocytes with the JNK inhibitor resulted in increased IL-4 mRNA expression (data not shown).

DISCUSSION

The results described above chart a novel signaling pathway that leads to down-regulation of Jun/AP-1 activity in T cells in response to TCR and CD28 engagement by accelerating the turnover of JunB and c-Jun (Figure 7). Previous studies have indicated that AP-1 proteins play an important role in induction of T cell cytokine genes both by direct binding to AP-1 sites in their promoters and by enabling NF-AT proteins to recognize low affinity sites (Rao *et al.* (1997) Annu Rev Immunol 15, 707-747; Rooney *et al.* (1995) Immunity 2, 473-483). Furthermore, the transcriptional activities of both c-Jun and JunB were shown to be enhanced by JNK-mediated phosphorylation in T cells (Li *et al.* (1999) Embo J 18, 420-432; Su *et al.* (1994) Cell 77, 727-736). These findings led to the expectation that c-Jun and JunB and the pathway that regulates their activity, namely the JNK pathway, play a positive role in expression of T cell cytokine genes. This expectation was partially borne out for JunB, whose transgenic overexpression was shown to increase the expression of Th2 cytokine genes (Li *et al.* (1999) Embo J 18, 420-432), while its ablation

severely attenuated their induction (Hartenstein *et al.* (2002) Embo J 21, 6321-6329). Interestingly, however, the manipulation of JunB levels did not alter the expression of Th1 cytokines, such as IFN-g, or IL-2, despite the presence of AP-1 sites in their promoters (Penix *et al.* (1996) J Biol Chem 271, 31964-31972; Serfling *et al.* (1989) Embo J 8, 465-473). Curiously, however, the ablation of both JNK1 and JNK2, the protein kinases responsible for enhanced JunB activity (Li *et al.* (1999) Embo J 18, 420-432), was found to dramatically enhance, rather than inhibit, the production of Th2 cytokines (Dong *et al.* (2000) Nature 405, 91-94). Similar results were obtained by ablation of MKK7 (JNKK2), the MAP2K responsible for JNK activation in T cells (Dong *et al.* (2000) Nature 405, 91-94), although one study attributed the hyperproliferation of MKK7-deficient lymphocytes to decreased p16 expression (Sasaki *et al.* (2001) J Exp Med 194, 757-768), an affect not seen in Mekk1KD T cells. However, none of these studies provided a mechanistic explanation for the negative effect of the JNK pathway on Th2 cytokine expression, neither were the targets for this inhibitory effect identified. We now show that elimination of MEKK1 catalytic activity in thymocytes and mature T cells results in eventually the same effect on cell proliferation and Th2 cytokine production as produced by elimination of JNK1/2 or MKK7 activities. We provide both biochemical and genetic evidence that the inhibitory effect of MEKK1 on T cell proliferation and Th2 cytokine gene expression is mediated via JNK, as already suggested by the analysis of JNK1/2-and MKK7-deficient T cells (Dong *et al.* (2000) Nature 405, 91-94). Most importantly, we have identified the biochemical pathway through which the MEKK1-dependent JNK cascade exerts this negative activity. We show that thymocytes lacking MEKK1 catalytic activity accumulate both c-Jun and JunB, while they continue to express normal levels of other transcription factors involved in Th2 cytokine expression or JunD. The accumulation of c-Jun and JunB is shown to be due to reduced JNK activity as well as their decreased degradation. The effect of the MEKK1/JNK cascade on Jun protein turnover and cytokine production is essentially identical to that of the E3 ubiquitin ligase Itch. Previous studies revealed that Itchy mice, expressing an inactive form of Itch, overproduce Th2 cytokines and accumulate c-Jun and JunB in their T cells, while expressing normal levels of JunD or other transcription factors (Fang *et al.* (2002) Nat Immunol 3, 281-287). Most

interestingly, we identify a very efficient and nearly quantitative interaction between MEKK1 and Itch and show that reduced MEKK1 or JNK activity results in accelerated Itch turnover, while slowing the turnover of c-Jun and JunB.

Our results suggest that the primary function of the MEKK1/JNK cascade in T cells undergoing robust activation brought about by strong co-stimulation of both TCR and CD28 is to enhance the Itch-dependent degradation of important regulatory proteins, such as JunB. As JunB is required for Th2, but not Th1, cytokine production (Hartenstein *et al.* (2002) Embo J 21, 6321-6329), the overall effect of this pathway is to attenuate the production of IL-4, the critical Th2 cytokine (Paul *et al.* (1994) Cell 76, 241-251), and thereby decrease the polarization of naïve Th cells towards the Th2 phenotype (Figure 7).

As far as we know, this pathway provides the first example where ubiquitin-dependent protein degradation is regulated not via substrate protein phosphorylation but through the modulation of a substrate-specific E3 ligase. In other experiments, we found that the Itch-dependent ubiquitination of c-Jun is not affected by elimination of the JNK phosphorylation sites and that T cells from c-JunAA mice, in which serines 63 and 73 of c-Jun [the JNK phosphorylation sites (Smeal *et al.* (1991) Nature 354, 494-496)], were replaced with alanines (Behrens *et al.* (1999) Nat Genet 21, 326-329), express lower levels of c-Jun relative to WT counterparts (data not shown). These findings are consistent with both the previous suggestion that JNK-mediated phosphorylation can lead to reduced ubiquitination-dependent c-Jun degradation (Fuchs *et al.* (1996) Oncogene 13, 1531-1535; Musti *et al.* (1997) Science 275, 400-402), and with the substrate specificity of E3 ligases with WW domains, such as Itch, which is not directed toward phospho-epitopes (Joazeiro *et al.* (2000) Science 289, 2061-2062; D. Fang, unpublished data). Although the exact mechanism through which the MEKK1/JNK cascade regulates Itch turnover remains to be identified, we note that it is cell type specific, as primarily lymphocytes, but neglibigly in other cell types, of $Mekk1^{KD}$ mice exhibit reduced Itch expression.

We propose that the major biological function of the pathway through which MEKK1/JNK activation leads to accelerated Itch-dependent JunB turnover in T cells is to attenuate the polarization of naïve Th cells towards the Th2 phenotype in response to output

from the TCR and co-stimulatory receptors. It was previously demonstrated that the extent and duration of TCR occupancy by antigen affects the differentiation of naïve Th cells into the Th1 and Th2 effector cell types or modulate the expression levels of critical Th1 and Th2 cytokines (Boyton *et al.* (2002) Trends Immunol 23, 526-529; Constant *et al.* (1995) J Exp Med 182, 1591-1596; Itoh *et al.* (1997) J Exp Med 186, 757-766; Kuchroo *et al.* (1995) Cell 80, 707-718; Lanzavecchia *et al.* (2000) Science 290, 92-97). Most relevant to our findings were the observations that high antigen doses polarize naïve Th cells towards the Th1 phenotype, while low doses of the same peptide antigen preferentially induced Th2 differentiation (Constant *et al.* (1995) J Exp Med 182, 1591-1596) and that T cells that express lower affinity TCRs may be more prone to differentiate towards the Th2 phenotype (Boyton *et al.* (2002) Trends Immunol 23, 526-529). There are also suggestions that co-engagement of CD28 may inhibit Th2 differentiation, while promoting Th1 differentiation (Kuchroo *et al.* (1995) Cell 80, 707-718). The mechanism through which the potency or duration of T cell activation affects effector functions is unknown. We suggest that one such mechanism may entail MEKK1/JNK activation, which promotes Itch-dependent degradation of JunB, thereby inhibiting IL-4 production and formation of Th2 cells. Given that JNK activation requires co-engagement of both TCR and CD28 (Su *et al.* (1994) Cell 77, 727-736) or large Ca2+ transients (Dolmetsch *et al.* (1997) Nature 386, 855-858), it is certainly feasible that the MEKK1/JNK cascade is involved in interpreting the potency of the TCR signal. Therefore, strong JNK activation produced by intense or prolonged T cell stimulation is expected to inhibit differentiation to Th2 phenotype, a prediction that is entirely consistent with available results. It is noteworthy that differentiated Th2 cells exhibit reduced Ca2+ transients (Sloan-Lancaster *et al.* (1997) J Immunol 159, 1160-1168) and thus may be less capable of mounting a strong JNK activation response. Given our suggestion that strong or prolonged JNK activation may inhibit Th2 differentiation, these cells may have been selected to become Th2 cells because of their reduced Ca2+ signaling capacity.

It is not intended to convey that any of these cells, proteins, molecules, pathways, complexes, antibodies, and receptors have primarily one function. Physiological pathways are in flux, for example activation pathways, and not usually isolated from each other. There are

several activation pathways leading towards cytokine production that overlap with several other pathways leading towards cell survival, proliferation, differentiation, activated cell death and apoptosis.. For example, cytokine production pathways overlap in that one protein, such as MEKK1, under some circumstances contributes to increasing type 2 cytokine levels, such as when destabilized, while under other circumstances MEEK1 will contribute to decreasing type 2 cytokine levels such as when intact. The same is true for JNK1 and ITCH. Often these counteractive results are found in different cells types. Furthermore, compensatory mechanisms and/or redundancies within type 2 cytokine production pathways often counteract and/or mask the ability of any one protein to contribute to either T2 cytokine or T1 cytokine production. Therefore, the present invention is unique in clearly showing the contributions of MEEK1, JNK1 and ITCH towards increasing type 2 cytokine levels and the value thereof.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof. In the experimental disclosure which follows, the following abbreviations apply: M (molar); mM (millimolar); μM (micromolar); nM (nanomolar); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); gm (grams); mg (milligrams); μg (micrograms); pg (picograms); L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); °C (degrees Centigrade).

EXAMPLE 1

Materials and Methods The following is a description of exemplary materials and methods that were used in subsequent Examples.

Sources of mice:

It is not intended to limit the source of mice. In one embodiment, mice were obtained by personal donations (for example, L. Chang for provided $Jnk1^{-/-}$ mice and E. D. Gallagher provided anti-MEKK1 antibodies). In one embodiment, mice were obtained by engineering

and breeding mice (for example, $Mekk1^{KD/KD}$ mutant mice were generated by standard procedures from $Mekk1^{KD}$ ES cells, in which the MEKK1 kinase domain was replaced with a b-galactosidase coding cassette (Xia *et al.*, 2000). Chimeric mice were generated by injection of $Mekk1^{KD}$ ES cells into C57BL/6 blastocysts. As used herein, the term "blastocysts" and "blastocyst cells" refers to a preimplantation embryo of 30-150 cells. A blastocysts contains a layer of specialized cells made up of trophoblasts which function to attach to the uterine wall and form the placenta. Inside the trophoblast layer is the undifferentiated inner cell mass. As used herein, the terms "ES" and "embryonic stem cells" refers to cells that contains the potential to differentiate into every cell type within a developing embryo. Embryonic Stem cells are derived from the inner cell mass of a blastocyst.

$Mekk1^{KD/KD}$ mice were generated by intercrossing $Mekk1^{+/KD}$ mice. $Jnk1^{-/-}$ mice were previously described (Sabapathy *et al.*, 2001)).

As used herein, the term "chimeric" refers to a composite of genetically distinct individuals, (*e.g.* following cell implantation from one animal into another, an allogeneic bone marrow graft, *etc.*).

As used herein, the terms "transgenic" and "mutant" when used in reference to an animal or to a cell refers to an animal or cell, respectively, which comprises one or more cells that contain a transgene, or whose genome has been altered by the introduction of a transgene. Transgenic cells, tissues and plants may be produced by several methods including the introduction of a "transgene" comprising nucleic acid (usually DNA) into a target cell or integration of the transgene into a chromosome of a target cell by way of human intervention, such as by the methods described herein. In one embodiment, knockout mice were of the C57BL/6 background. As used herein, the term "knockout" refers to a deletion or deactivation or ablation of a gene or deficient gene in a mouse or other laboratory animal or any cells in an animal. When said knockout includes the germ cells, subsequent breeding can create a line of animals that are incapable of or produce significantly less of said gene product. As used herein, the term "transgenic" or "mutant" when used in reference to a cell refers to a cell which contains a transgene, or whose genome has been altered by the introduction of a transgene.

Flow cytometry and cell isolation:

Single-cell suspensions of thymi and spleens were prepared from 6-8-week-old mice. Splenic CD4+ and CD8+ T cells were prepared using magnetic beads (Miltenyi Biotech). Monoclonal FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 antibodies (PharMingen) were used for cell staining. Analyses were performed on a FACScan flow cytometer (Becton Dickinson) using CELL Quest software.

Plasmids and transfections:

Full-length or kinase domain-truncated MEKK1 cDNAs were described (Xia *et al.* (2000) Proc Natl Acad Sci U S A 97, 5243-5248). The ubiquitin expression vector with a Myc or HA epitope tag was also described (Fang *et al.* (2001) J Biol Chem 276, 4872-4878). c-Jun and JunB cDNAs were HA tagged as described and subcloned into the mammalian expression vector pEFneo (Invitrogen) (Fang *et al.* (2002) Nat Immunol 3, 281-287). 293T cells were cultured and transfected with various plasmids as described (Fang *et al.* (2001) J Biol Chem 276, 4872-4878).

Thymocyte proliferation and survival assays:

For proliferation assays, purified thymocytes (2′105 cells/well) were cultured in round bottom 96-well plates (Costar) precoated with anti-CD3 antibody (BD Pharmingen) in the absence or presence of soluble anti-CD28 antibody (BD Pharmingen). After 3 days, cultures were pulsed for 6 hrs with 1 mCi [^{3}H] thymidine (NEN) per well and cells were harvested. [^{3}H] thymidine incorporation was measured by scintillation counting. When indicated, either recombinant IL-4 or neutralizing anti-IL-4 antibody (R&D Systems) was added to the cultures prior to addition of [^{3}H] thymidine. For analysis of cell death, freshly isolated thymocytes were cultured in 24-well plates (Costar) precoated with anti-CD3 with or without soluble anti-CD28. At various time points after stimulation, viable thymocytes were identified by trypan blue exclusion. Apoptotic cells were identified by annexin-V and propidium iodide (Clontech) staining.

Kinase assays and immunoblotting:

JNK1 was immunoprecipitated from 30 mg cell lysate with anti-JNK1 antibody (333, PharMingen) and its kinase activity measured by an immunecomplex kinase assay with GST-c-Jun(1-79) as a substrate (Xia *et al.* (2000) Proc Natl Acad Sci U S A 97, 5243-5248). c-Jun phosphorylation was quantitated using a PhosphoImager (Bio-Rad) and gel loading was normalized by immunoblotting with a monoclonal antibody against JNK1/2 (666, PharMingen). ERK activation was examined by immunoblotting of gel separated whole cell extracts (100 mg) with an antibody specific for phospho-ERK (Cell Signaling). Polyclonal antibody against MEKK1 was prepared as described (Gallagher *et al.* (2002) J Biol Chem 277, 45785-45792). Antibodies against c-Jun, JunB, GATA3, c-Maf, NFATc1, NFATc2, p16, and p65 (RelA) were all from Santa Cruz Biotechnology. Polyclonal antibody against Itch was prepared as described (Qiu *et al.* (2000) J Biol Chem 275, 35734-35737).

RNA analysis:

Total RNA from thymocytes was prepared using RNeasy kit (Qiagen). RNase protection analysis was performed using the RiboQuant multi-probe system (Pharmingen). Mouse cytokine multi-probe sets mCK1 and mJun/Fos were transcribed and radiolabeled using MAXIscript *in vitro* transcription kit (Ambion). Total RNA (2 mg) was hybridized to the probes and analyzed following the manufacturer's protocols. For real time PCR analysis, total thymocyte RNA (2 mg) was used to synthesize cDNA by SuperScript First-Strand Synthesis System (Invitrogen). cDNA products were resuspended in 200 ml of dH2O and 5 ml cDNA was used in a Real-Time PCR assay. PCR amplifications were performed in a total volume of 25 ml containing cDNA template, cytokine-specific primers, and Master Green SYBR Green reagent (Roche). Real-Time PCR reactions were performed in triplicates using an ABI Prism 7700 Sequence Detector (Applied Biosystems). The cytokine-specific primers used in this study were described (Giulietti *et al.* (2001) Methods 25, 386-401). Cyclophilin A mRNA was used for normalization.

Pulse-chase experiments:

[^{35}S] labeling and pulse-chase experiments were performed as described (Fang *et al.* (2002) Nat Immunol 3, 281-287). Purified thymocytes were cultured in 24-well plates in Dulbecco's modified Eagle's medium (DMEM) lacking methionine and cysteine. Cells were stimulated with anti-CD3 (10 mg/ml) + anti-CD28 (1 mg/ml) for 24 hrs and then pulse-labeled for 1 hr by adding 100 mCi/ml [^{35}S] methionine and [^{35}S] cysteine (Amersham Biosciences). Cells were then chased for different times with cold amino acids and cell lysates were immunoprecipitated with anti-c-Jun, anti-JunB, anti-RelA (p65) (all from Santa Cruz) and anti-Itch (Qiu *et al.* (2000) J Biol Chem 275, 35734-35737). The immunecomplexes were separated by SDS-PAGE and labeled proteins were detected by autoradiography.

Chromatin immunoprecipitation assays (ChIP):

ChIP assays were carried out as described. (Saccani *et al.* (2002) Genes Dev 16, 2219-2224). Polyclonal antibodies against c-Jun (H-79) and JunB (C-11) were from Santa Cruz. The primers for amplifying the IL-4 promotor region were 5'-GTTGCTGAAACCAAGGGAAA-3' and 5'-TGAAAGGCCGATTATGGTGT-3'. The primers for amplifying the IL-4 coding region were 5'-TCAACCCCCAGCTAGTTGTC-3' and 5'-AAATATGCGAAGCACCTTGG-3'.

EXAMPLE 2

Hyperproliferation of Mekk1^{KD} mutant T cells

A. Thymocytes from WT and Mekk1^{KD} mice were incubated with the indicated concentrations (mg/ml) of anti-CD3 or anti-CD3 + anti-CD28 for 72 hrs. Cell proliferation was measured by [^{3}H] thymidine incorporation. Results are averages of 6 experiments. (FIG. 1A)

B. Thymocytes from WT and Mekk1^{KD} mice were cultured with 5 microg/ml of anti-CD3 with or without 0.5 mg/ml of anti-CD28. At the indicated times, cell viability was

determined by trypan blue staining. Values represent the mean proportion of viable thymocytes relative to untreated cultures (100%) in 3 separate experiments. (FIG. 1B)

C. CD4+ and CD8+ splenic T cells from WT and Mekk1^{KD} mice were treated with 10 mg/ml anti-CD3 and 1 mg/ml anti-CD28 for 48 hrs. Cell proliferation was measured as above. Results are averages of 3 experiments. (FIG. 1C)

EXAMPLE 3

Reduced JNK activity results in increased thymocyte proliferation

A. WT and Mekk1^{KD} thymocytes were incubated with anti-CD3 (10 mg/ml) and anti-CD28 (1 mg/ml). At the indicated times, JNK activity was measured by an immunecomplex kinase assay using GST-c-Jun(1-79) as the substrate. Phosphorylated c-Jun was detected by autoradiography and quantitated using a PhosphorImager. The level of immunoprecipitated JNKs was determined by immunoblotting. This experiment was repeated several times with similar results. (FIG. 2A)

B. WT and Mekk1^{KD} thymocytes were stimulated as above. At the indicated times, ERK activation was examined by immunoblotting with an antibody against phosphorylated ERK. The same membrane was reprobed with a general anti-ERK antibody. (FIG. 2B)

C. Thymocytes were incubated with indicated concentrations (mg/ml) of anti-CD3 and anti-CD28 in the presence of increasing concentrations (mM) of the JNK inhibitor SP600125 (SP) for 72 hrs. Cell proliferation was measured by [^{3}H] thymidine incorporation. (FIG. 2C)

D. Thymocytes from Jnk1$^{+/-}$ and Mekk1$^{+/KD}$ Jnk1$^{+/-}$ mice were treated with indicated concentrations (mg/ml) of anti-CD3 with or without anti-CD28 for 72 hrs. Cell proliferation was measured as above. (FIG. 2D)

EXAMPLE 4

Enhanced Th2 cytokine production by Mekk1^{KD} thymocytes

A. WT and Mekk1^{KD} thymocytes were stimulated with anti-CD3 (5 mg/ml) and anti-CD28 (0.5 mg/ml) for 48 hrs. Expression of cytokine mRNAs was measured by RNase protection.

The levels of ribosomal protein L32 and GAPDH mRNAs were used as loading controls. (FIG. 3A)

B. WT and Mekk1^{KD} thymocytes were stimulated as above. The levels of various cytokines mRNAs were quantitated by Real-time PCR and normalized to the level of cyclophilin A mRNA. (FIG. 3B)

C. Mekk1^{KD} thymocytes were incubated with indicated concentrations (mg/ml) of anti-CD3 or anti-CD3 + anti-CD28 for 72 hrs in the presence of increasing amounts of anti-IL-4. Cell proliferation was measured by [^{3}H] thymidine incorporation. (FIG. 3C)

D. WT thymocytes were incubated with the indicated concentrations (mg/ml) of anti-CD3 or anti-CD3 + anti-CD28 for 72 hrs in the presence of increasing amounts of IL-4. Cell proliferation was measured as above. (FIG. 3D)

EXAMPLE 5

Upregulation of JunB and c-Jun protein levels in Mekk1^{KD} thymocytes

A. WT and Mekk1^{KD} thymocytes were stimulated with anti-CD3 (5 mg/ml) + anti-CD28 (0.5 mg/ml) for 24 hrs. Cell extracts were prepared and the levels of various transcription factors were measured by immunoblotting and quantitated using a PhosphoImager. The relative levels of the different proteins are indicated. (FIG. 4A)

B. WT and Mekk1^{KD} thymocytes were stimulated as described above. mRNA levels of Jun family members were measured by RNase protection. (FIG. 4B)

C. WT and Mekk1^{KD} thymocytes were stimulated as above. Cells were collected and recruitment of c-Jun and JunB to the indicated regions of the IL-4 gene was examined by ChIP experiments. (FIG. 4C)

EXAMPLE 6

MEKK1 promotes ubiquitin-dependent degradation of c-Jun and JunB

A. WT and Mekk1^{KD} thymocytes were pulse-labeled with medium containing [^{35}S] cysteine and methionine for 1 hr in the presence of anti-CD3 (5 microg/ml) + anti-CD28 (0.5 microg/ml). Cells were chased with medium containing non-labeled amino acids for the indicated times. Cell lysates were immunoprecipitated with various antibodies as indicated,

separated by SDS-PAGE and subjected to autoradiography. The amounts of radioactivity were quantitated using a PhosphoImager. This experiment was repeated twice with similar results. (FIG. 5A)

B. The data in panel A were quantitated and normalized. The relative amounts of each [^{35}S]-labeled protein at t=0 was considered to be 100%. (FIG. 5B)

C. 293T cells were transiently transfected with plasmids encoding HA-tagged c-Jun or JunB, WT MEKK1 or a kinase domain-truncated MEKK1 (mt), and Myc-Ub. c-Jun and JunB were immunoprecipitated and Ub conjugation was examined with anti-Myc. The same membrane was reprobed for JunB and c-Jun. Data are shown for JunB. This experiment was repeated twice with similar results. (FIG. 5C)

EXAMPLE 7

MEKK1/JNK signaling pathway regulates Itch and Jun turnover

A. WT and Mekk1^{KD} thymocytes were cultured in the presence of anti-CD3 (5 mg/ml) + anti-CD28 (0.5 mg/ml) for 24 hrs. The levels of Itch and actin were examined was measured by immunoblotting. (FIG. 6A)

B. Thymocytes were lysed and immunoprecipitated with indicated antibodies. The immunecomplexes were separated by SDS-PAGE and immunoblotted with anti-MEKK1 and anti-Itch. The positions of MEKK1 and IgG heavy chain are indicated. This experiments was repeated 3 times with similar results. (FIG. 6B)

C. WT and Mekk1^{KD} thymocytes were pulse-labeled with [^{35}S] cysteine and methionine for 1 hr in the presence of anti-CD3 (5 mg/ml) + anti-CD28 (0.5 mg/ml). Cells were chased with nonradioactive amino acids for the indicated times, at which they were lysed and Itch expression levels were determined by radioimmunoprecipitation. The levels of [^{35}S]-labeled Itch were quantitated using a PhosphoImager. (FIG. 6C)

D. WT and Mekk1^{KD} thymocytes were stimulated as above for 24 hrs in the absence or presence (0.5 mM) of the JNK inhibitor SP600125 (SP). Cell lysates were prepared and the levels of the different proteins were examined by immunoblotting. (FIG. 6D)

E. Thymocytes were stimulated for 5 min in the absence or presence (0.5 mM) of the JNK inhibitor (SP). JNK activity was measured by an immunecomplex kinase assay.

Phosphorylated c-Jun was detected by autoradiography and quantitated using a PhosphoImager. (FIG. 6E)

F. 293T cells were transfected with plasmids encoding HA-Ub and Myc-tagged c-Jun or JunB. Cells were incubated with (1.0 mM) or without the JNK inhibitor (SP) for 24 hrs. The Jun proteins were immunoprecipitated and Ub conjugation was examined as described above. (FIG. 6F)

An important role in protein ubiquitination is played by the E3 ubiquitin protein ligases, which are responsible for substrate recognition (Hershko *et al.* (1998) Annu Rev Biochem 67, 425-479).

EXAMPLE 8

MEKK1/JNK signaling pathway negatively and positively regulates JunB stability and activity in response to T cell stimulation

Engagement of TCR and CD28 co-stimulatory receptor results in activation of the MEKK1 to MKK4/MEKK7 to JNK1/2 signaling cascade. Transcriptional activity of JunB is enhanced by this pathway through direct phosphorylation by JNKs and leads to transcription of IL-4, a key cytokine for Th2 cells. On the other hand, the MEKK1/JNK pathway regulates the stability of the E3 ubiquitin protein ligase Itch, which promotes the polyubiquitination and degradation of JunB. This pathway, which dominates under intense T cell activation conditions, inhibits Th2 cytokine production and Th2 differentiation.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the invention.

The MEKK1-JNK Cascade Inhibits Th2 Cytokine Production through Itch-mediated Jun Degradation in Response to T cell Receptor Activation

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Summary

T cell receptor (TCR) engagement results in activation of several transcription factors, including AP-1, leading to increased cytokine production. It was suggested that the intensity or duration of the activating stimulus bias the cytokine expression profile of T helper (Th) cells, but the underlying biochemical mechanism is unknown. Although TCR engagement can increase Jun/AP-1 transcriptional activity through JNK-mediated phosphorylation, we found that it also promotes Jun protein turnover via a mechanism dependent on JNK and its upstream activator MEKK1, which associates with the E3 ubiquitin-ligase Itch. TCR activation stimulates Itch activity in a JNK-dependent manner and inhibition of either MEKK1 or JNK slows down the turnover of JunB and increases Th2 cytokine production and differentiation. This pathway may function to attenuate Th2 cytokine production following intense T cell stimulation.

Introduction

Engagement of T cell receptor (TCR) along with co-stimulatory receptors, such as CD28, activates several signaling pathways that stimulate the activity of various transcription factors involved in production of T cell cytokines (Crabtree and Clipstone, 1994; Weiss and Littman, 1994). These transcription factors include NF-AT and NF-κB family members, as well as members of the AP-1 family (Crabtree and Clipstone, 1994; Nolan, 1994; Rao et al., 1997; Weiss and Littman, 1994). In addition to cytokine gene induction, it was suggested that the intensity and duration of the signal generated by engagement of TCR and co-stimulatory receptors can modulate the differentiation of naïve T helper (Th) cells into the Th1 and Th2 effector subsets (Boyton and Altmann, 2002; Constant et al., 1995; Itoh and Germain, 1997; Kuchroo et al., 1995;

Lanzavecchia and Sallusto, 2000). While the mechanisms involved activation of T cell cytokine genes are relatively well understood (Murphy et al., 2000), the biochemical mechanisms by which TCR-generated signals modulate the spectrum of cytokine gene expression are unknown. Nonetheless, it was observed that Th1 and Th2 cells exhibit fundamental differences in Ca^{2+} signaling (Gajewski et al., 1990; Sloan-Lancaster et al., 1997).

Some of the transcription factors required for Th cell polarization and differentiation were identified (Murphy et al., 2000). These include T-bet which is required for commitment to the Th1 phenotype (Szabo et al., 2000) and GATA3 (Zheng and Flavell, 1997), c-Maf (Ho et al., 1996), and JunB (Hartenstein et al., 2002; Li et al., 1999) that induce the Th2 phenotype. JunB is a member of the AP-1 family of transcription factors, which also includes c-Jun and JunD, as well as c-Fos, FosB, Fra1 and Fra2 and several other proteins (Angel and Karin, 1991). AP-1 activity is subject to complex regulation, in which members of the mitogen activated protein kinase (MAPK) family play an important role both by regulating the expression of *Jun* and *Fos* genes, as well as by modulating the transcriptional activity of their protein products through direct phosphorylation (Karin, 1995). Amongst the key regulators of AP-1 activity are the Jun N-terminal kinases (JNKs), which in addition to the Jun proteins (Kallunki et al., 1996), also phosphorylate other transcription factors (Minden and Karin, 1997). Importantly, in T cells the JNKs are primary members of the MAPK family whose activation in response to TCR engagement is strongly potentiated by occupancy of the co-stimulatory receptor CD28 (Su et al., 1994). These findings suggested that JNK may be involved in interpretation of co-stimulatory signals or the

intensity of T cell activation. TCR-mediated JNK activation depends on Ca^{2+} signaling (Werlen et al., 1998).

In addition to direct binding to several cytokine gene promoters, such as that of *IL-4* (Li et al., 1999; Rooney et al., 1995), AP-1 proteins are required for cooperative binding of NF-AT proteins to low affinity sites (Rao et al., 1997; Rooney et al., 1995). While all three Jun proteins are expressed in T cells, it was found that differentiation towards the Th2 phenotype is accompanied by up-regulation of JunB, whose ectopic expression can polarize naïve Th cells towards the Th2 phenotype (Li et al., 1999). Gene disruption experiments confirmed the critical role of JunB in expression of Th2 cytokines (Hartenstein et al., 2002), which include IL-4, IL-5, IL-10 and IL-13 (Murphy et al., 2000; Paul and Seder, 1994). JunB can directly activate the *IL-4* promoter in cooperation with c-Maf and this activity is strongly potentiated by JNK-mediated JunB phosphorylation (Li et al., 1999). Despite the ability of JNK to enhance JunB (and c-Jun) transcriptional activity, the loss of both JNK1 and JNK2 strongly enhances, rather than attenuates, the generation of Th2 effector cells (Dong et al., 2000). These results suggest that the JNKs may have a complex and rather enigmatic role in regulation of JunB or other transcription factors required for expression of Th2 cytokines.

Another protein involved in the regulation of Jun protein activity or expression in T cells is the E3 ubiquitin ligase Itch (Fang et al., 2002). *Itchy* mutant mice, which express an inactive form of Itch, exhibit increased expression of Th2 cytokines as well as elevated levels of c-Jun and JunB in their T cells (Fang et al., 2002). Itch is a member of the HECT domain group of E3 ubiquitin-protein ligases (Qiu et al., 2000).

These proteins are believed to be constitutively active enzymes that recognize their substrates via a WW domain, which unlike the F boxes used by members of the SCF group of ubiquitin-protein ligases, do not recognize phospho-epitopes (Ciechanover et al., 2000; Joazeiro and Hunter, 2000). Itch was shown to interact with both JunB and c-Jun and stimulate their polyubiquitination in an in vitro system (Fang et al., 2002).

To further understand the role of the JNK pathway in regulation of AP-1 and Jun activity in T cells, we analyzed the role of the MAPK kinase (MAP2K) kinase (MAP3K) MEKK1 in TCR-mediated JNK activation and gene expression. MEKK1 is one of the most potent activators of the JNK cascade identified (Minden and Karin, 1997). Here we show that MEKK1 is the major activator of the JNK cascade in response to TCR and CD28 co-ligation. Interestingly, disruption of MEKK1 catalytic activity or JNK inhibition results in marked overproduction of Th2 cytokines while having little effect on Th1 cytokines. Furthermore, like inactivation of Itch, the loss of MEKK1 catalytic activity results in stabilization of c-Jun and JunB. We show that MEKK1 physically interacts with Itch and that MEKK1-dependent JNK activation is required for stimulation of Itch catalytic activity in response to T cell activation. We also show that strong T cell activation via TCR and CD28 results in enhanced JunB turnover and that this enhancement is dependent on MEKK1 and JNK activities. Based on these results we suggest that TCR and CD28 co-ligation results in activation of the MEKK1 to JNK cascade, which has two opposing effects on JunB and c-Jun. While enhancing their transcriptional activity through direct phosphorylation, it also leads to their increased turnover and eventual degradation through enhanced Itch activity. This

can provide a biochemical mechanism through which the strength of output from TCR and co-stimulatory receptors modulates Th cell differentiation.

Results

Reduced JNK activation in *Mekk1*$^{\Delta KD}$ mutant T cells results in hyper-responsiveness

Although the roles of JNK1 and JNK2, as well as the upstream kinase MKK7, in thymocyte and peripheral T cell activation were examined, some of the results were rather controversial (Dong et al., 2000; Sabapathy et al., 2001; Sasaki et al., 2001) and as discussed above inconsistent with results obtained for the JNK target JunB (Hartenstein et al., 2002; Li et al., 1999). We wanted to determine whether MEKK1 is the MAP3K responsible for JNK activation in response to TCR engagement and if so examine its function in T cells. We first examined the effect of the *Mekk1*$^{\Delta KD}$ mutation on activation of JNK and other MAPKs in response to TCR and CD28 engagement. Loss of MEKK1 catalytic activity resulted in a considerable decrease in JNK activation following stimulation of thymocytes with anti-CD3 + anti-CD28 (Figure 1A). By contrast, ERK activation was not affected. p38 MAPK was not activated by these stimuli in either WT or *Mekk1*$^{\Delta KD}$ thymocytes (data not shown). Similar results were obtained when splenic T cells were examined (data not shown).

Next we examined the effect of the *Mekk1*$^{\Delta KD}$ mutation on T cell function in response to TCR activation. Loss of MEKK1 catalytic activity had little effect on thymocyte numbers and differentiation into the CD4 and CD8 double and single positive classes (unpublished results). However, *Mekk1*$^{\Delta KD}$ thymocytes were hyper-responsive to TCR engagement with anti-CD3 antibody and this hyper-responsiveness was strongly

augmented by ligation of the co-stimulatory CD28 receptor (Figure 1B). Proliferation in response to PMA and ionomycin, pharmacological stimuli that bypass TCR and CD28, was not affected (data not shown). The increased proliferative response was not due to decreased activation-induced death, as cell viability was not changed in *Mekk1*$^{\Delta KD}$ thymocytes (unpublished results). In addition, the expression levels of several cell death regulators were unchanged (data not shown).

To examine whether the effect of MEKK1 on thymocyte proliferation is mediated through JNK, we bred *Jnk1*$^{-/-}$ mice with *Mekk1*$^{\Delta KD}$ mice. Importantly, *Mekk1*$^{+/\Delta KD}$*Jnk1*$^{+/-}$ thymocytes exhibited a pronounced hyper-responsiveness to TCR and CD28 engagement in comparison to either *Jnk1*$^{+/-}$ (Figure 1C) or *Mekk1*$^{+/\Delta KD}$ (data not shown) thymocytes. To confirm that loss of JNK activity causes TCR hyper-responsiveness, we also used the small molecule JNK inhibitor SP600125 (Bennett et al., 2001). Stimulation of WT thymocytes with different concentrations of anti-CD3 + anti-CD28 in the presence of increasing amounts of SP600125, which inhibited JNK activity (see below), resulted in at least 2-fold increase in [^{3}H] thymidine incorporation (Figure 1D). On the other hand, the p38 inhibitor SB202190 (Lee et al., 1994) did not produce such an effect (data not shown). Similar, but less pronounced, hyper-responsiveness was observed in peripheral *Mekk1*$^{\Delta KD}$ T cells (Figure 1E), whereas the rate of cell death was comparable between WT and *Mekk1*$^{\Delta KD}$ splenic T cells (data not shown).

Increased Th2 cytokine production by *Mekk1*$^{\Delta KD}$ T cells

To determine the mechanism by which reduced JNK and MEKK1 activity leads to thymocyte and T cell hyper-responsiveness, we compared the pattern of TCR and

downstream effector protein tyrosine phosphorylation between WT and mutant cells and found negligible obvious differences (data not shown). We also compared the levels of various cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors, including p16, between WT and mutant thymocytes and found negligible major differences in their expression (data not shown). Next, we considered the role of cytokine gene expression in the hyper-responsive phenotype of *Mekk1*$^{\Delta KD}$ T cells, as cytokines play crucial roles in regulating T cell proliferation and differentiation (Murphy et al., 2000; Paul and Seder, 1994; Smith et al., 1979). Indeed, *Mekk1*$^{\Delta KD}$ thymocytes were found to express elevated levels of IL-4 and IL-13 mRNAs but close to normal levels of interferon γ (IFNγ) mRNA after stimulation with anti-CD3 + anti-CD28 (Figure 2A). Unstimulated thymocytes expressed background levels of these mRNAs (data not shown). In addition to ribonuclease (RNase) protection analysis, we used a more sensitive Real-time PCR assay to quantitate the levels of these and other mRNAs in CD4$^+$ T cells from WT and *Mekk1*$^{\Delta KD}$ mice after 24 hrs stimulation with anti-CD3 + anti-CD28. While expression of IL-2 or IFNγ mRNAs was not dramatically affected, *Mekk1*$^{\Delta KD}$ T cells expressed up to 5-fold more IL-4, IL-5, IL-10 and IL-13 mRNAs (Figure 2B). Up-regulation of these mRNAs was also observed in *Mekk1*$^{\Delta KD}$ thymocytes using Real-time PCR (data not shown).

The cytokine mRNAs that are overexpressed in *Mekk1*$^{\Delta KD}$ CD4$^+$ T cells are characteristic of the Th2 effector cell type (Murphy et al., 2000; Paul and Seder, 1994). Therefore, MEKK1 catalytic activity may be important in differentiation of primary T cells into the Th1 and Th2 effector subsets. To test this hypothesis, we cultured naïve WT or *Mekk1*$^{\Delta KD}$ CD4$^+$ T cells under Th1- or Th2-polarization conditions for 7 days.

The cells were restimulated with anti-CD3 + anti-CD28, and the outcome was examined by intracellular staining for IL-4 and IFNγ. Deletion of the MEKK1 catalytic domain strongly enhanced the generation of IL-4$^+$ Th2 cells but had negligible effect on generation of IFNγ$^+$ Th1 cells (Figure 2C).

To determine whether the elevated Th2 response of *Mekk1*$^{\Delta KD}$ CD4$^+$ T cells is IL-4-dependent, we crossed *IL-4*$^{-/-}$ mice (Metwali et al., 1996) with *Mekk1*$^{\Delta KD}$ mice. We found that the elevated production of all Th2-type cytokines seen in *Mekk1*$^{\Delta KD}$*IL-4*$^{+/+}$ T cells was reduced to normal in *Mekk1*$^{\Delta KD}$*IL-4*$^{-/-}$ cells (Figure 2D). To determine whether increased IL-4 production was also responsible for the hyperproliferation of *Mekk1*$^{\Delta KD}$ T cells, we examined the effect of a neutralizing anti-IL-4 antibody as well as the *IL-4* gene deletion. Incubation with anti-IL-4, but not control anti-IgG, markedly reduced the proliferation of *Mekk1*$^{\Delta KD}$ thymocytes elicited by anti-CD3 in the absence or presence of anti-CD28 (Figure 2E and data not shown). Similar results were obtained upon deletion of the *IL-4* gene (data not shown). In addition, addition of exogenous IL-4 to WT thymocytes strongly potentiated their proliferative response (data not shown).

Elevated c-Jun and JunB protein accumulation in *Mekk1*$^{\Delta KD}$ T cells

The molecular regulation of IL-4, IL-5, and IL-13 gene expression was recently reviewed (Murphy et al., 2000). Multiple transcription factors, including NF-AT, GATA-3, c-Maf, and JunB, are involved in IL-4 gene induction (Murphy et al., 2000; Paul and Seder, 1994). JunB (Li et al., 1999), as well as its relative c-Jun (Hibi et al., 1993; Kallunki et al., 1996), are targets for JNK-mediated phosphorylation. Given the pivotal role of IL-4 in Th2 differentiation and in the *Mekk1*$^{\Delta KD}$ T cell phenotype, we

examined the effect of the mutation on the levels and subcellular distribution of these transcription factors. Both c-Jun and JunB were elevated in activated thymocytes and $CD4^+$ T cells from *Mekk1*$^{\Delta KD}$ mice, while expression of JunD, another family member, was unchanged (Figure 3A). *Mekk1*$^{\Delta KD}$ cells, also expressed normal levels of GATA-3, c-Maf, NF-ATc1 and NF-ATc2. In addition, nuclear translocation of NF-ATs appeared to be normal in *Mekk1*$^{\Delta KD}$ T cells (data not shown).

Surprisingly, despite the change in protein levels, the levels of *c-Jun* and *JunB* mRNAs were not different between WT and mutant thymocytes (Figure 3B). A possible mechanism through which MEKK1 and JNK can modulate c-Jun and JunB levels is by promoting their turnover. To examine this point, we conducted pulse-chase experiments. In WT thymocytes, newly synthesized c-Jun and JunB proteins were degraded with half-lives ($t_{1/2}$) of 59 and 78 min, respectively (Figure 3C). Both proteins were significantly more stable in *Mekk1*$^{\Delta KD}$ thymocytes, where their $t_{1/2}$ were extended to 109 and 280 min, respectively. By contrast, the turnover of RelA(p65) was unaltered between WT and *Mekk1*$^{\Delta KD}$ cells. To test whether the effect of MEKK1 on Jun protein turnover was mediated through JNK, we used two different JNK inhibitors. Incubation of activated WT thymocytes with either SP600125 or JNKI-1, a peptide inhibitor based on the c-Jun docking site for JNK (Borsello et al., 2003; Kallunki et al., 1996), resulted in about a 4-fold increase in c-Jun and JunB expression (Figure 3D). Negligible such effects were observed upon incubation with the p38 inhibitor SB202190 (data not shown). The effect of the JNK inhibitors on Jun protein levels correlated with their effects on JNK activation. In addition to the effects on Jun expression, treatment of $CD4^+$ T cells with either JNK inhibitor resulted in increased IL-4 mRNA induction (data not shown).

The MEKK1-JNK pathway enhances the E3 ubiquitin-ligase activity of Itch

Degradation of many short-lived transcription factors is controlled by ubiquitin-dependent proteolysis (Hershko and Ciechanover, 1998). An important role in protein ubiquitination is played by E3 ubiquitin protein ligases, which are responsible for substrate recognition (Hershko and Ciechanover, 1998). Elevated expression of c-Jun and JunB and increased Th2 cytokine production were recently described in T cells from *Itchy* mutant mice, that express an inactive form of Itch, an HECT domain E3 ubiquitin protein ligase (Fang et al, 2002). The similarities with the phenotype of *Mekk1*$^{\Delta KD}$ T cells raised the possibility that MEKK1-directed JunB (or c-Jun) turnover is Itch-dependent and that the MEKK1-JNK cascade may control Itch activity. Importantly, we found that Itch and MEKK1 exhibited very efficient and nearly quantitative interaction in both thymocytes and splenic T cells (Figure 4A and data not shown). Although the expression levels of Itch and MEKK1 were not modulated, the interaction between Itch and MEKK1 was enhanced after TCR activation (Figure 4B). Yet, the loss of MEKK1 or JNK activity did not prevent the formation of the MEKK1-Itch complex (data not shown).

Next, we wished to know whether the MEKK1-JNK signaling cascade regulates the E3 ligase activity of Itch or its ability to recognize c-Jun and JunB. We first immunoprecipitated Itch from anti-CD3 + anti-CD28 stimulated and unstimulated WT and *Mekk1*$^{\Delta KD}$ T cells. Ubiquitination of Itch was analyzed by an in vitro ubiquitination assay. As shown by the slower migrating bands detected by immunoblotting with anti-ubiquitin antibody, the ubiquitination of Itch (presumably due to autoubiquitination) was stimulated by TCR activation (Figure 4C). However, this effect was markedly reduced in

Mekk1$^{\Delta KD}$ T cells. Ubiquitination of Itch was E1 and E2 dependent as omission of E1, E2, or ATP prevented Itch ubiquitination (data not shown). To further examine whether the MEKK1-JNK cascade regulates Itch E3 activity towards Jun proteins, purified GST-c-Jun fusion protein was incubated with immunoprecipitated Itch in the presence of E1, E2, and ubiquitin. As revealed by immunoblotting with anti-c-Jun antibody, Itch isolated from primary T cells mediated polyubiquitination of c-Jun in a manner dependent on E1 and E2 (data not shown). The E3 activity of Itch towards c-Jun was enhanced upon TCR and CD28 co-ligation of WT T cells, while such an increase in Itch activity was not found in *Mekk1*$^{\Delta KD}$ T cells (Figure 4D). In addition, we examined the effect of the JNK inhibitor SP600125 on the regulation of Itch activity. Both Itch autoubiquitination and c-Jun polyubiquitination were significantly reduced in anti-CD3 + anti-CD28 stimulated T cells pretreated with the JNK inhibitor (Figure 4E). Similar results were also obtained with the peptide inhibitor of JNK (data not shown). We conclude that the E3 ubiquitin ligase activity of Itch is strongly enhanced in response to TCR and CD28 activation of T cells and that the MEKK1-JNK pathway plays a major role in this response.

We further examined whether MEKK1 and JNK enhance Itch-dependent ubiquitination of c-Jun or JunB in living cells. Due to difficulties in detecting endogenously ubiquitinated proteins and simplifying the interpretation of the results, we used transiently transfected cells. 293T cells were transfected with plasmids encoding hemagglutinin (HA)-tagged ubiquitin, Myc-tagged JunB or c-Jun, WT or kinase domain-truncated MEKK1, similar to the MEKK1 polypeptide expressed in *Mekk1*$^{\Delta KD}$ cells, and either WT or a catalytically inactive Itch mutant (Qiu et al, 2000). Cell lysates were immunoprecipitated with anti-Myc antibody, and ubiquitination of c-Jun and JunB was

monitored by immunoblotting of gel-separated proteins with anti-HA. Ectopic expression of WT MEKK1 promoted the polyubiquitination of both c-Jun and JunB in a manner highly dependent on expression of WT Itch (Figure 5A, and data not shown for c-Jun). Overexpression of kinase-deleted MEKK1 did not enhance the extent of Itch-dependent Jun polyubiquitination. The inactive Itch mutant acted in a dominant-negative manner and reduced both basal and MEKK1-stimulated Jun polyubiquitination. These data indicate that MEKK1 can enhance Itch-mediated Jun ubiquitination in cells. To better understand the role of MEKK1 in enhancement of Jun ubiquitination, 293T cells were cotransfected as above with HA-tagged ubiquitin, Myc-JunB, Itch as well as WT and mutant versions of MEKK1. The C433A mutation lies in the PHD domain of MEKK1 and reduces its kinase activity (Figure 5B). The F1442A mutation lies within the kinase domain of MEKK1 and has a small negative effect on its kinase activity whereas the adjacent T1381A mutation results in complete loss of kinase activity (Figure 5B). The expression levels of all three mutants are similar to those of WT MEKK1 (E. G., unpublished results). As before, WT MEKK1 strongly enhanced the polyubiquitination of JunB, while less potent enhancement was produced by the F1443 mutant (Figure 5B). On the other hand, hardly any increase in JunB polyubiquitination was seen upon expression of the C433A and T1381A mutants. These data show that the ability of MEKK1 to activate JNK correlates with its ability to enhance Itch-dependent Jun ubiquitination.

To confirm the role of JNK in Itch-dependent Jun ubiquitination, we cotransfected 293T cells with HA-ubiquitin, Myc-tagged c-Jun or JunB, WT or mutant Itch, and either active (WT) or inactive (mt) JNKK2-JNK1 fusion proteins (Zheng et al., 1999).

Overexpression of the active JNKK2-JNK1 fusion protein enhanced Itch-dependent polyubiquitination of both c-Jun and JunB, while the inactive JNKK2-JNK1 fusion protein did not display such an activity (Figure 5C). Consistent with this, we also found that treatment with the JNK inhibitor reduced the extent of Itch-induced c-Jun and JunB polyubiquitination in transfected cells (Figure 5D). Since JNK phosphorylates c-Jun (Hibi et al., 1993) and possibly JunB (Li et al., 1999), we investigated whether JNK-dependent phosphorylation of the Jun proteins modulated their Itch-induced ubiquitination. 293T cells were transfected with HA-ubiquitin, Itch, MEKK1, and WT and mutant versions of c-Jun that either lacked one or both of the known JNK phosphorylation sites (Smeal et al., 1991). Consistent with the independence of HECT domain ligases of substrate phosphorylation (Ciechanover et al., 2000; Joazeiro and Hunter, 2000), the N-terminal phosphorylation of c-Jun by JNK had negligible effect on the extent of Itch-induced polyubiquitination (Figure 5E). Thus, JNK-mediated phosphorylation enhances Jun ubiquitination through enhancement of Itch catalytic activity.

Costimulation of T cells accelerates JunB degradation

We next examined the biological effects of TCR activation on JunB expression. $CD4^+$ T cells were incubated with either anti-CD3 alone or anti-CD3 + anti-CD28 for several days. The mRNA and protein levels of JunB were examined at different time points and the ratios of protein to RNA were compared. Both conditions induced the expression of JunB mRNA and protein in naïve $CD4^+$ T cells (Figure 6A, B). Although the level of JunB mRNA at day 1 was higher in cells treated with anti-CD3 + anti-CD28

than in cells exposed to anti-CD3 alone, the protein levels were quite similar. Interestingly, after 4 days of stimulation cells exposed to anti-CD3 + anti-CD28 hardly contained any JunB protein, while JunB was readily detected in cells exposed to anti-CD3 alone (Figure 6A). By contrast, the mRNA levels of JunB were very similar after 4 days of stimulation with either anti-CD3 or anti-CD3 + anti-CD28 (Figure 6B). These results suggest that although costimulation of T cells results in induction of JunB, it accelerates the turnover of the protein relative to cells exposed to anti-CD3 alone.

Next, we compared JunB mRNA and protein accumulation in $CD4^+$ primary T cells from WT and *Mekk1*$^{\Delta KD}$ mice stimulated with anti-CD3 + anti-CD28. Whereas the levels of JunB mRNA were very similar in T cells of the two genotypes, the levels of JunB protein, already after one day of stimulation, were higher in the mutant cells (Figure 6C, D). Most striking, were the differences in JunB protein levels seen after 4 days of stimulation. These results, strongly suggest that the accelerated turnover of JunB in co-stimulated T cells is dependent on MEKK1 catalytic activity.

Discussion

The results described above chart a novel signaling pathway that leads to down-regulation of Jun/AP-1 activity in T cells in response to TCR and CD28 engagement by accelerating the turnover of JunB and c-Jun (Figure 7). Previous studies revealed that AP-1 proteins play an important role in induction of T cell cytokine genes both by direct binding to AP-1 sites in their promoters and by enabling NF-AT proteins to recognize low affinity sites (Rao et al., 1997; Rooney et al., 1995). Furthermore, the transcriptional activities of both c-Jun and JunB were shown to be enhanced by JNK-mediated

phosphorylation in T cells (Li et al., 1999; Su et al., 1994). These findings led to the expectation that c-Jun and JunB and the pathway that regulates their activity, namely the JNK pathway, play a positive role in expression of T cell cytokine genes. This expectation was partially borne out for JunB, whose transgenic overexpression was shown to increase the expression of Th2 cytokine genes (Li et al., 1999), while its ablation severely attenuated their induction (Hartenstein et al., 2002). Interestingly, however, the manipulation of JunB levels did not alter the expression of Th1 cytokines, such as IFNγ, or IL-2, despite the presence of AP-1 sites in their promoters (Penix et al., 1996; Serfling et al., 1989). Curiously, however, the ablation of both JNK1 and JNK2, the protein kinases thought to be responsible for enhanced JunB activity (Li et al., 1999), was found to dramatically enhance, rather than inhibit, the production of Th2 cytokines (Dong et al., 2000). Similar results were obtained by ablation of MKK7 (JNKK2), the MAP2K responsible for JNK activation in T cells (Dong et al., 2000), although one study attributed the hyperproliferation of *Mkk7*-deficient lymphocytes to decreased p16 expression (Sasaki et al., 2001), an effect not seen in $Mekk1^{KD}$ T cells. However, none of these studies provided a mechanistic explanation for the negative effect of the JNK pathway on Th2 cytokine expression, neither were the targets for this inhibitory effect identified.

We now show that elimination of MEKK1 catalytic activity or JNK inhibition result in essentially the same effect on T cell proliferation and Th2 cytokine production as seen after elimination of JNK1/2 or MKK7 or overexpression of JunB. We provide both biochemical and genetic evidence that the inhibitory effect of MEKK1 on T cell proliferation and Th2 cytokine gene expression is indeed mediated via JNK. Most

importantly, we have identified the biochemical pathway through which MEKK1-dependent JNK activation exerts this negative regulatory activity. We show that $CD4^+$ T cells lacking MEKK1 catalytic activity or T cells treated with JNK inhibitors accumulate both c-Jun and JunB, while they continue to express normal levels of other transcription factors involved in Th2 cytokine expression or JunD. The accumulation of c-Jun and JunB is shown to be due to their decreased degradation. This effect of disruption of the MEKK1-JNK cascade on Jun protein turnover and cytokine production is essentially identical to that of inactivation of the E3 ubiquitin ligase Itch. Previous studies revealed that *Itchy* mice, expressing an inactive form of Itch, overproduce Th2 cytokines and accumulate c-Jun and JunB in their T cells, while expressing normal levels of JunD or other transcription factors (Fang et al., 2002). Most importantly, our work shows that T cell activation enhances the E3 ligase activity of Itch and that JNK activation, which is compromised in $Mekk1^{\Delta KD}$ T cells, is required for this enhancement. As far as we know, this is the first report demonstrating enhanced Itch activity in response to TCR and CD28 co-ligation. Until now, Itch was thought to be a constitutively active ubiquitin ligase.

Our results suggest that the primary function of the MEKK1-JNK cascade in T cells undergoing robust activation brought about by strong co-stimulation of both TCR and CD28 is to enhance the Itch-dependent degradation of important regulatory proteins, such as JunB. As JunB is required for Th2, but not Th1, cytokine production (Hartenstein et al., 2002), the overall effect of this pathway is to attenuate the production of IL-4, the critical Th2 cytokine (Debonneville et al., 2001; Paul and Seder, 1994), and thereby decrease the polarization of naïve Th cells towards the Th2 phenotype (Figure 7). Importantly, MEKK1 catalytic activity is not required for JunB induction and the strength

of T cell activation has little effect on the level of *JunB* mRNA. Almost as much *JunB* mRNA accumulates after engagement of TCR alone as after co-ligation of TCR and CD28 (Figure 6).

Our data provide a physiologically relevant example that ubiquitin-dependent protein turnover can be regulated via substrate protein phosphorylation but also through modulation of the catalytic activity of a substrate-specific E3 ligase. Regulation of the HECT domain E3 ligase function by phosphorylation has been previously addressed in studies on Nedd4-2, a homolog of Itch (Debonneville et al., 2001). However, in that case Nedd4-2 phosphorylation inhibited its substrate binding activity. Our results provide evidence that the catalytic activity of Itch is positively regulated. We also found that the Itch-dependent ubiquitination of c-Jun is not affected by elimination of the JNK phosphorylation sites and that T cells from *c-Jun*^{AA} mice, in which serines 63 and 73 of c-Jun [the JNK phosphorylation sites (Smeal et al., 1991)], were replaced with alanines (Behrens et al., 1999), express lower levels of c-Jun relative to WT counterparts (data not shown). These findings are consistent with both the previous suggestion that JNK-mediated phosphorylation can lead to reduced ubiquitin-dependent c-Jun degradation (Fuchs et al., 1996; Musti et al., 1997), and with the known substrate specificity of HECT domain E3 ligases, such as Itch, which is not directed toward phospho-epitopes (Joazeiro and Hunter, 2000). We also show that disruption of the PHD domain of MEKK1 abolishes Itch-dependent Jun ubiquitination. Similar to results obtained in previous studies on the involvement of MEKK1 in ERK degradation (Lu et al., 2002), our data suggest that the PHD domain of MEKK1 has an important role in the regulation of

protein ubiquitination. However, the major effect of this domain could be mediated through modulation of MEKK1 kinase activity.

We propose that the major biological function of the pathway through which MEKK1/JNK activation enhances Itch activity and leads to accelerated Itch-dependent JunB turnover in T cells is to attenuate the polarization of naïve Th cells towards the Th2 phenotype following strong T cell stimulation, as occurs after co-engagement of TCR and CD28. It was previously demonstrated that the extent and duration of TCR occupancy by antigen affects the differentiation of naïve Th cells into the Th1 and Th2 effector cell types or can modulate the expression levels of critical Th1 and Th2 cytokines (Boyton and Altmann, 2002; Brogdon et al., 2002; Constant et al., 1995; Itoh and Germain, 1997; Kuchroo et al., 1995; Lanzavecchia and Sallusto, 2000). For instance, high antigen doses polarize naïve Th cells towards the Th1 phenotype, while low doses of the same peptide antigen preferentially induce Th2 differentiation (Brogdon et al., 2002; Constant et al., 1995). In addition, T cells that express lower affinity TCRs may be more prone to differentiate towards the Th2 phenotype (Boyton and Altmann, 2002). There are also suggestions that co-engagement of CD28 may inhibit Th2 differentiation, while promoting Th1 differentiation (Kuchroo et al, 1995). The mechanism through which the potency or duration of T cell activation affects effector functions is unknown. We suggest that one such mechanism may entail MEKK1/JNK activation, which promotes Itch-dependent degradation of JunB, thereby inhibiting IL-4 production and formation of Th2 cells. Given that JNK activation requires co-engagement of both TCR and CD28 (Su et al., 1994) or large Ca^{2+} transients (Dolmetsch et al., 1997), it is certainly feasible that the MEKK1-JNK cascade may interpret the potency of the TCR signal. Therefore,

strong JNK activation produced by intense or prolonged T cell stimulation is expected to inhibit differentiation towards the Th2 phenotype, a prediction that is entirely consistent with current results. It is noteworthy that differentiated Th2 cells exhibit reduced Ca^{2+} transients (Sloan-Lancaster et al., 1997) and thus may be less capable of mounting a strong JNK activation response. Given our suggestion that strong or prolonged JNK activation may inhibit Th2 differentiation, these cells may have been selected to become Th2 cells because of their reduced Ca^{2+} signaling capacity.

Experimental Procedures

Mice

Mekk1$^{\Delta KD}$ mice were generated by standard procedures from *Mekk1*$^{\Delta KD}$ ES cells, in which the MEKK1 kinase domain was deleted and replaced with a β galactosidase coding cassette (Xia et al., 2000). *Jnk1*$^{-/-}$ mice were previously described (Sabapathy et al., 2001). *IL-4*$^{-/-}$ mice were purchased from the Jackson Laboratory. Similar results were obtained by analyzing the *Mekk1*$^{\Delta KD}$ mutation in a mixed 129×BL6 or pure BL6 background.

Flow cytometry and T cell isolation

Single-cell suspensions of thymi and spleens were prepared from 6-8-week-old mice. Thymic or splenic $CD4^+$ and $CD8^+$ T cells were prepared using magnetic beads (Miltenyi Biotech). Monoclonal FITC-conjugated anti-CD8, PE-conjugated anti-CD4, anti-IL4 and anti-IFNγ antibodies (PharMingen) were used for cell staining. Analyses

were performed on a FACScan flow cytometer (Becton Dickinson) using CELL Quest software.

T cell proliferation assays

For proliferation assays, purified thymocytes or $CD4^+$ or $CD8^+$ splenic T cells (2×10^5 cells/well) were cultured in round bottom 96-well plates (Costar) precoated with anti-CD3 antibody (BD Pharmingen) in the absence or presence of soluble anti-CD28 antibody (BD Pharmingen). After 3 days, cultures were pulsed for 6 hrs with 1 μCi [^{3}H] thymidine (New England Nuclear) per well and cells were harvested. [^{3}H] thymidine incorporation was measured by scintillation counting. When indicated, either recombinant IL-4 or neutralizing anti-IL-4 antibody (R&D Systems) were added prior to [^{3}H] thymidine labeling.

Th cell differentiation and activation

$CD4^+$ T cells were purified from spleens of WT or *Mekk1*$^{\Delta KD}$ mice. Primary T cells were incubated with anti-CD3 (10 μg/ml) and mitomycin C (50 μg/ml)-treated WT antigen presenting cells (APC) in the presence of anti–IL-4 (2 μg/ml) + IL-12 (0.1 μg/ml) for Th1 differentiation or anti–IFNγ (1 μg/ml) + IL-4 (50 ng/ml) for Th2 differentiation. IL-2 (20 U/ml) was added to both the Th1 and Th2 cultures. After 7 days, Th subsets were collected, washed and restimulated with anti-CD3 (10 μg/ml) + anti-CD28 (1 μg/ml). Intracellular staining for IL-4 and IFNγ was performed using the cytofix/cytoperm kit (BD BioSciences).

Kinase assays and immunoblotting

JNK1 was immunoprecipitated from cell lysates (30 μg proteins) with anti-JNK1 antibody (333, PharMingen) and its activity measured by an immunecomplex kinase assay with GST-c-Jun(1-79) as a substrate (Xia et al., 2000). c-Jun phosphorylation was quantitated using a PhosphoImager (Bio-Rad) and gel loading was normalized by immunoblotting with anti-JNK1/2 monoclonal antibody (666, PharMingen). ERK activation was examined by immunoblotting gel-separated whole cell extracts (100 μg) with anti-phospho-ERK antibody (Cell Signaling). Polyclonal antibody against MEKK1 was prepared as described (Gallagher et al., 2002). Antibodies against c-Jun, JunB, GATA3, c-Maf, NFATc1, NFATc2, p16, and p65(RelA) were all from Santa Cruz Biotechnology. Anti-Itch antibody was described (Qiu et al., 2000). The levels of various proteins were quantitated by BioRad Quantity One software.

RNA analysis

Total RNA from thymocytes and $CD4^+$ T cells was prepared using RNeasy kit (Qiagen). RNase protection analysis was performed using RiboQuant multi-probe (Pharmingen). Mouse cytokine multi-probe sets mCK1 and mJun/Fos were transcribed and radiolabeled using the MAXIscript kit (Ambion). Total RNA (2 μg) was hybridized to the probes and analyzed according to the manufacturer's protocols. For Real-time PCR analysis, total RNA (2 μg) was used to synthesize cDNA by SuperScript First-Strand Synthesis System (Invitrogen). cDNA products were resuspended in 200 μl of dH_2O and 5 μl cDNA samples were used in a Real-Time PCR assay, in a total volume of 25 μl containing cDNA, cytokine-specific primers, and Master Green SYBR Green

reagent (Roche). Real-Time PCR reactions were performed in triplicates using an ABI Prism 7700 Sequence Detector (Applied Biosystems). The cytokine-specific primers used in this study were described (Giulietti et al., 2001). Cyclophilin A mRNA was used for normalization.

Pulse-chase experiments

[^{35}S] labeling and pulse-chase experiments were performed as described (Fang et al., 2002). Purified thymocytes were cultured in 24-well plates in Dulbecco's modified Eagle's medium (DMEM) lacking methionine and cysteine. Cells were stimulated with anti-CD3 (10 μg/ml) + anti-CD28 (1 μg/ml) for 24 hrs and then pulse-labeled for 1 hr by adding 100 μCi/ml [^{35}S] methionine and [^{35}S] cysteine (Amersham Biosciences). Cells were chased for different times with cold amino acids and cell lysates were immunoprecipitated with anti-c-Jun, anti-JunB, anti-RelA(p65) (all from Santa Cruz). Immunecomplexes were separated by SDS-PAGE and labeled proteins were detected by autoradiography.

Plasmids and transfections

Full-length or kinase domain-truncated MEKK1 cDNAs were described (Xia et al., 2000). The ubiquitin expression vector with a Myc or HA epitope tag was also described (Fang et al., 2001). The C433A, F1443A and T1381A of MEKK1 mutants were generated by PCR mutagenesis and subcloned into the pCMV expression vector. WT or kinase dead JNKK2-JNK1 fusion proteins were described (Zheng et al, 1999). c-Jun and JunB cDNAs were HA tagged and subcloned into the mammalian expression vector

pEFneo (Invitrogen). WT and catalytically inactive Itch expression vectors were described (Qiu et al, 2000). 293T cells were cultured and transfected with various plasmids as described (Fang et al., 2001).

In vitro ubiquitination assays

His-tagged ubiquitin, E1, and GST-Ubc7 (E2) were prepared as described (Joazeiro et al, 1999) and used in in vitro ubiquitination assays (Qiu, 2000). Ubiquitination reaction mixtures containing E1 (100 nM), Ubc7 (0.5 μM), ubiquitin (5 μM), ATP (2 mM), GST-c-Jun (2 μg), and immunoprecipitated Itch on Sepharose beads were incubated at 25^0C for 90 min. Anti-c-Jun antibody was used for immunoblotting. To examine Itch autoubiquitination, reactions were incubated in the absence of GST-c-Jun and analyzed by immunoblotting with an anti-ubiquitin or anti-Itch antibodies after re-precipitation of Itch. Omission of E1, E2, or ATP prevented Itch or c-Jun ubiquitination (data not shown).

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Figure Legends

Figure 1. Reduced JNK activity and increased T cell proliferation in *Mekk1*$^{\Delta KD}$ T cells.

A. WT and *Mekk1*$^{\Delta KD}$ thymocytes were incubated with anti-CD3 (10 μg/ml) + anti-CD28 (1 μg/ml). At the indicated times, JNK activity was measured by an immunecomplex kinase assay with GST-c-Jun(1-79) as the substrate. Phosphorylated c-Jun was detected by autoradiography and quantitated using a PhosphorImager. The level of immunoprecipitated JNKs was determined by immunoblotting. ERK activation was examined by immunoblotting with anti-phospho-ERK. The same membrane was reprobed with a general anti-ERK antibody.

B. WT and *Mekk1*$^{\Delta KD}$ thymocytes were incubated with the indicated concentrations (μg/ml) of anti-CD3 or anti-CD3 + anti-CD28 for 72 hrs. Cell proliferation was measured by [^{3}H] thymidine incorporation. Results are averages of 6 experiments.

C. *Jnk1*$^{+/-}$ and *Mekk1*$^{+/\Delta KD}$ *Jnk1*$^{+/-}$ thymocytes were treated as above and cell proliferation was examined. Results are averages of 3 experiments.

D. WT thymocytes were incubated with indicated concentrations (μg/ml) of anti-CD3 + anti-CD28 in the presence of increasing concentrations (μM) of the JNK inhibitor SP600125 (SP) for 72 hrs. Cell proliferation was measured as above. Results are averages of 3 experiments.

E. CD4$^+$ and CD8$^+$ splenic T cells from WT and *Mekk1*$^{\Delta KD}$ mice were treated with anti-CD3 (10 μg/ml) + anti-CD28 (1 μg/ml) for 48 hrs. Cell proliferation was measured as above. Results are averages of 3 experiments.

Figure 2. Enhanced Th2 cytokine production and skewed differentiation by *Mekk1*$^{\Delta KD}$ T cells.

A. WT and *Mekk1*$^{\Delta KD}$ thymocytes were stimulated with anti-CD3 (5 μg/ml) + anti-CD28 (0.5 μg/ml) for 48 hrs. Expression of cytokine mRNAs was measured by RNase protection. The levels of ribosomal protein L32 and GAPDH mRNAs were used as loading controls.

B. CD4$^+$ T cells from WT and *Mekk1*$^{\Delta KD}$ mice were stimulated with anti-CD3 (5 μg/ml) + anti-CD28 (0.5 μg/ml) for 24 hrs. The levels of cytokine mRNAs were quantitated by Real-time PCR and normalized to the level of cyclophilin A mRNA. The relative amounts of IL-4 mRNA in WT cells were given an arbitrary level of 1.0.

C. CD4$^+$ T cells from WT and *Mekk1*$^{\Delta KD}$ mice were cultured under Th1- or Th2-polarizing conditions for 7 days. Th subsets were restimulated with anti-CD3 (10 μg/ml) + anti-CD28 (1 μg/ml) for 6 hrs, and analyzed for cytokine expression by flow cytometry.

D. CD4$^+$ T cells from WT, *Mekk1*$^{\Delta KD}$, and *Mekk1*$^{\Delta KD}$*IL-4*$^{-/-}$ mice were treated with anti-CD3 (5 μg/ml) + anti-CD28 (0.5 μg/ml) for 24 hrs. The levels of cytokine mRNAs were quantitated by Real-time PCR and normalized to the level of cyclophilin A mRNA. The relative amounts of IL-4 mRNA in WT cells were given an arbitrary level of 1.0.

E. *Mekk1*$^{\Delta KD}$ thymocytes were incubated with indicated concentrations (μg/ml) of anti-CD3 or anti-CD3 + anti-CD28 for 72 hrs in the presence of increasing amounts of anti-IL-4 and cell proliferation was examined.

Figure 3. Inactivation of MEKK1 or JNK slows down JunB and c-Jun turnover.

A. Thymocytes and $CD4^+$ T cells from WT and *Mekk1*$^{\Delta KD}$ were stimulated with anti-CD3 + anti-CD28 for 24 hrs. Cell extracts were prepared and the levels of various transcription factors were measured by immunoblotting. The relative levels of the different proteins determined by densitometry are indicated.

B. WT and *Mekk1*$^{\Delta KD}$ thymocytes were stimulated as above. The mRNA levels of Jun family members and c-Fos were measured by RNase protection. The levels of GAPDH mRNA were used as loading control.

C. WT and *Mekk1*$^{\Delta KD}$ thymocytes were pulse-labeled with [^{35}S] cysteine and methionine for 1 hr in the presence of anti-CD3 + anti-CD28 after one day incubation with these antibodies. Cells were chased with excess non-labeled amino acids for the indicated times. Cell lysates were prepared and immunoprecipitated with various antibodies as indicated, separated by SDS-PAGE and analyzed by autoradiography. The amounts of radioactivity in each protein band were quantitated using a PhosphorImager. The relative amounts of each [^{35}S]-labeled protein at t=0 was considered to be 100%. The results are averages of 3 separate experiments.

D. WT and *Mekk1*$^{\Delta KD}$ $CD4^+$ T cells were stimulated as above in the absence or presence of the JNK inhibitor SP600125 (0.5 μM) or JNKI-1 (25 μM) for 24 hrs. Cell extracts were prepared and the levels of indicated proteins were measured by immunoblotting. JNK activity was measured by an immunecomplex kinase assay. The relative levels of the different proteins relative to actin and JNK1/2 levels are indicated.

Figure 4. The MEKK1-JNK cascade enhances the E3 ubiquitin ligase activity of Itch.

A. Non-stimulated WT T cells were lysed and immunoprecipitated with the indicated antibodies. The immunecomplexes were gel separated and immunoblotted with anti-MEKK1 or anti-Itch antibodies. The positions of MEKK1, Itch and IgG heavy chain are indicated.

B. WT T cells were stimulated with anti-CD3 (10 μg/ml) + anti-CD28 (1 μg/ml) for the indicated times. Cell extracts were prepared and the levels of MEKK1 and Itch were measured by immunoblotting. Cell lysates were also immunoprecipitated with anti-MEKK1 and immunoblotted with anti-Itch as above.

C. WT and *Mekk1*$^{\Delta KD}$ T cells were left unstimulated or stimulated as above for 15 min. Cell extracts were prepared and Itch was isolated by immunoprecipitation. Itch was incubated with ubiquitin, E1, E2, and ATP at 25°C for 90 min. After reprecipitation of Itch, reaction mixtures were separated by SDS-PAGE and immunoblotted with anti-ubiquitin antibody. The same membrane was reprobed with anti-Itch antibody. The positions of non-ubiquitinated and ubiquitinated Itch are indicated.

D. WT and *Mekk1*$^{\Delta KD}$ T cells were treated as above. Itch immunecomplexes were isolated as above and incubated with ubiquitin, E1, E2, ATP and purified GST-c-Jun at 25°C for 90 min. The reaction mixtures were separated by SDS-PAGE and immunoblotted with anti-c-Jun. The same membrane was reprobed with anti-Itch.

E. WT T cells were stimulated as above in the absence or presence of the JNK inhibitor SP600125 (1 μM) for 15 min. In vitro ubiquitination assays using immunoprecipitated Itch as the E3 ubiquitin ligase with or without GST-c-Jun as the substrate were performed and analyzed as above.

Figure 5. The MEKK1-JNK cascade promotes Itch-dependent c-Jun and JunB ubiquitination in living cells.

A. 293T cells were transiently transfected with plasmids encoding HA-tagged ubiquitin, Myc-tagged JunB, WT or a kinase domain-deleted MEKK1 (mt), and WT or catalytically inactive (mt) Itch. After 24 hrs, JunB was immunoprecipitated and gel separated, and ubiquitin conjugation was examined by immunoblotting with anti-HA antibody.

B. 293T cells were cotransfected with HA-ubiquitin, Myc-JunB, Itch and the indicated MEKK1 constructs. JunB ubiquitination was examined as above. JNK activation was examined by immunoblotting with an antibody against endogenous phopho-JNK1/2. The same membrane was reprobed with a general anti-JNK1/2 antibody.

C. 293T cells were cotransfected with HA-ubiquitin, Myc-tagged c-Jun or JunB, WT or catalytically inactive (mt) Itch, and WT or inactive JNKK2-JNK1 (mt) fusion proteins. Jun ubiquitination was examined as above.

D. 293T cells were transfected as above. Cells were incubated with (1.0 μM) or without the JNK inhibitor SP600125 for 24 hrs. Jun proteins were immunoprecipitated and their ubiquitination was examined as above.

E. 293T cells were transfected as above including WT and phosphorylation-deficient c-Jun constructs. c-Jun ubiquitination was analyzed as above. c-Jun phosphorylation was examined by immunoblotting with anti-phospho-c-Jun(S63) antibody. The same membrane was reprobed with a general anti-c-Jun antibody. Very little c-Jun ubiquitination was seen in the absence of Itch (see panel A).

Figure 6. Co-stimulation of T cells enhances JunB turnover.

A. $CD4^+$ T cells were incubated with anti-CD3 (10 μg) alone or anti-CD3 (10 μg) + anti-CD28 (1 μg). At the indicated times (days), cell extracts were prepared and the levels of JunB were examined by immunoblotting.

B. $CD4^+$ T cells were stimulated as above. Levels of JunB protein were quantitated by immunoblotting, whereas *JunB* mRNA levels were quantitated by Real-time PCR. The relative amounts of JunB protein or mRNA at day 0 were given an arbitrary value of 1.0. Results are averages of 3 experiments done in duplicates.

C. WT and $Mekk1^{\Delta KD}$ $CD4^+$ T cells were stimulated with anti-CD3 + anti-CD28. At the indicated time points, cell extracts were prepared and JunB expression examined as above.

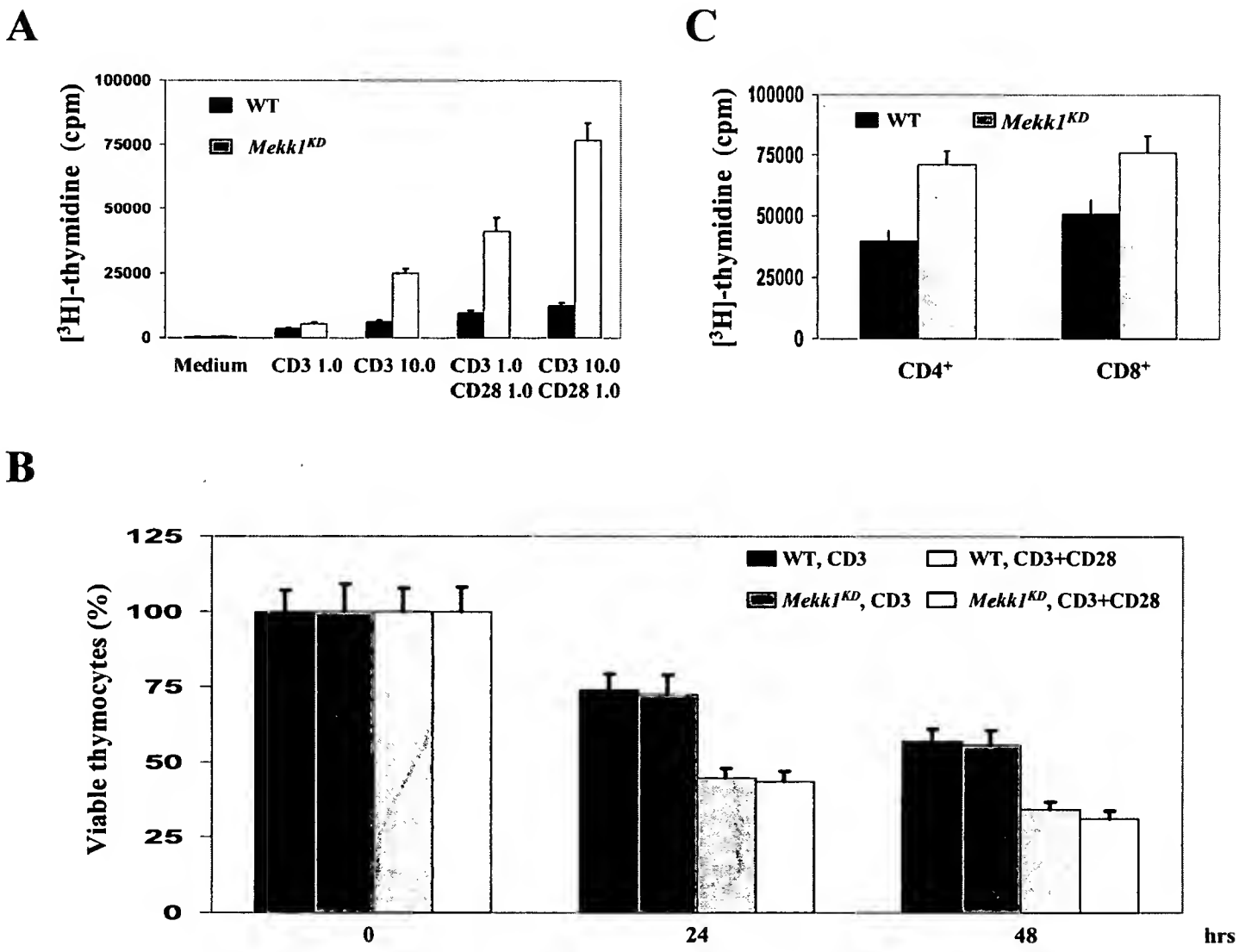
D. WT and $Mekk1^{\Delta KD}$ $CD4^+$ T cells were treated as above and the levels of JunB mRNA and protein were examined. The results are averages of 3 experiments done in duplicates.

Figure 7. JNK-dependent Itch activation modulates JunB turnover according to the strength of the T cell activating stimulus.

A model summarizing our results and illustrating a mechanism through which the MEKK1-JNK signaling pathway can modulate the extent of Th2 differentiation. The MEKK1-JNK signaling cascade regulates JunB turnover and thereby modulates Th2 cell differentiation according to the strength of the T cell activating stimulus by enhancing Itch activity. Co-stimulation of T cells with anti-CD3 + anti-CD28 (B) results in more JNK activity and accelerates the turnover of JunB relative to cells treated with anti-CD3 alone (A). Enhanced turnover of JunB attenuates the expression of IL-4 and negatively affects Th2 differentiation. Either stimulus results in initial induction of JunB.

ABSTRACT

The present invention relates to altering the levels of Th2 cytokine production, and in particular, biasing the cytokine expression profile towards Th2 cytokine production through mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1), the screening of agents that increase Th2 cytokine production, and the treatment of Th1 associated autoimmune diseases *in vivo*. In one embodiment, the present invention relates to agents including but not limited to reducing the activity of MEKK1, leading to increased levels of Th2 cytokine production.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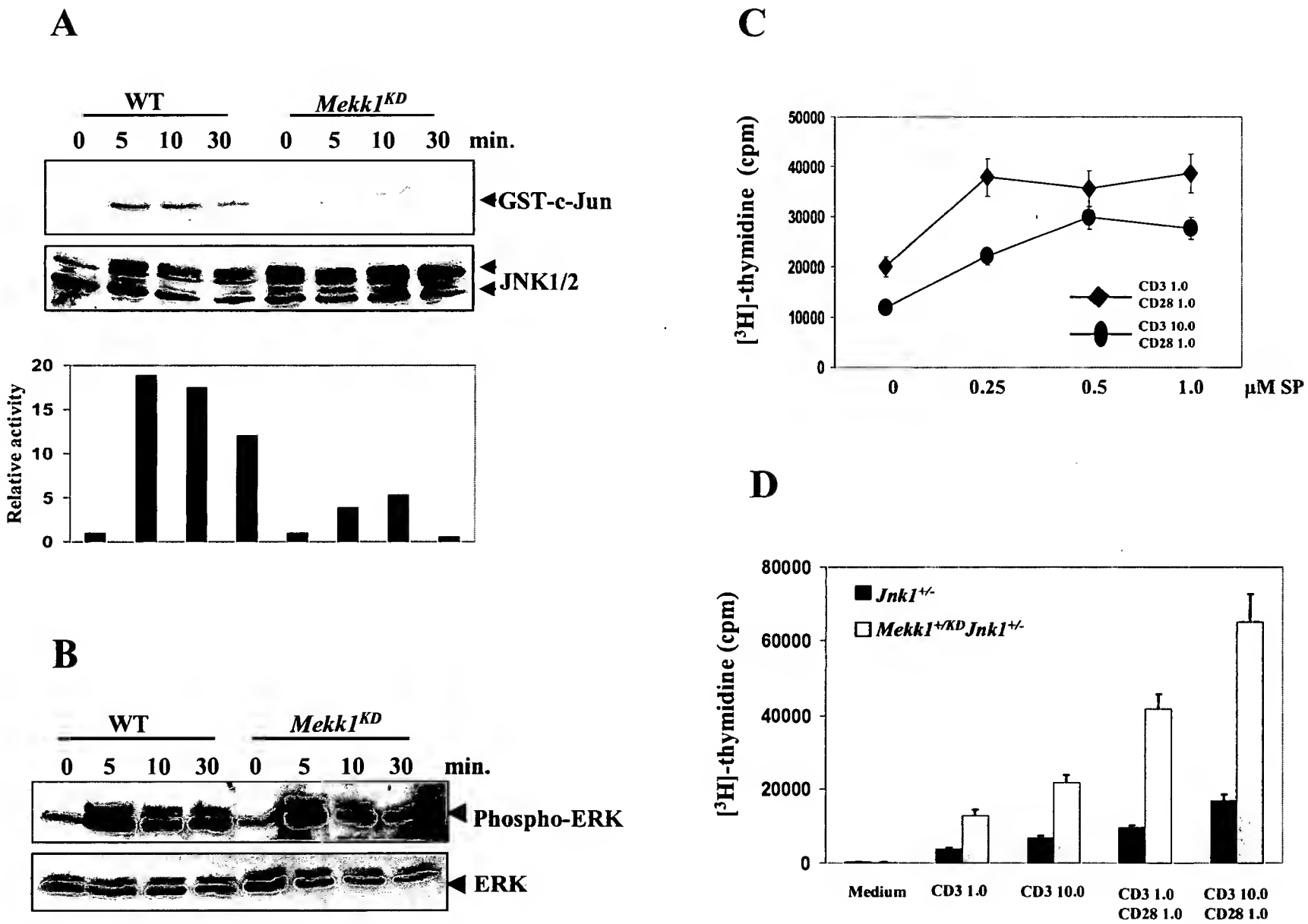
100000
75000
50000
25000
0
[3H]-thymidine (cpm)
WT
Mekk1KD
Medium
CD3 1.0
CD3 10.0
CD3 1.0 CD28 1.0
CD3 10.0 CD28 1.0
C
100000
75000
50000
25000
0
[3H]-thymidine (cpm)
WT
Mekk1KD
CD4+
CD8+
B
125
100
75
50
25
0
Viable thymocytes (%)
WT, CD3
WT, CD3+CD28
Mekk1KD, CD3
Mekk1KD, CD3+CD28
0
24
48
hrs

Fig. 1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WT
Mekk1KD
0 5 10 30 0 5 10 30 min.
GST-c-Jun
JNK1/2
Relative activity
20
15
10
5
0
B
WT
Mekk1KD
0 5 10 30 0 5 10 30 min.
Phospho-ERK
ERK
C
[3H]-thymidine (cpm)
50000
40000
30000
20000
10000
0
CD3 1.0
CD28 1.0
CD3 10.0
CD28 1.0
0 0.25 0.5 1.0 µM SP
D
[3H]-thymidine (cpm)
80000
60000
40000
20000
0
Jnk1+/-
Mekk1+/KDJnk1+/-
Medium
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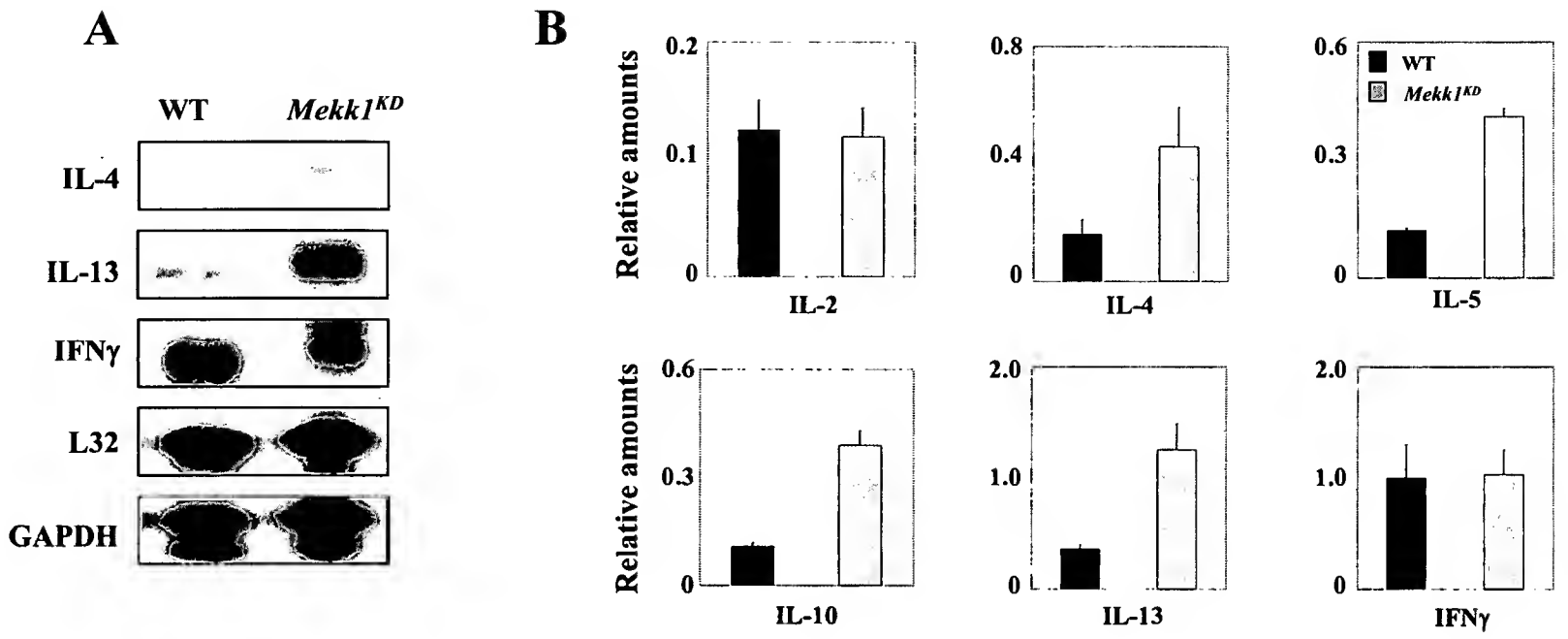
CD3 10.0
CD3 1.0 CD28 1.0
CD3 10.0 CD28 1.0

Fig. 2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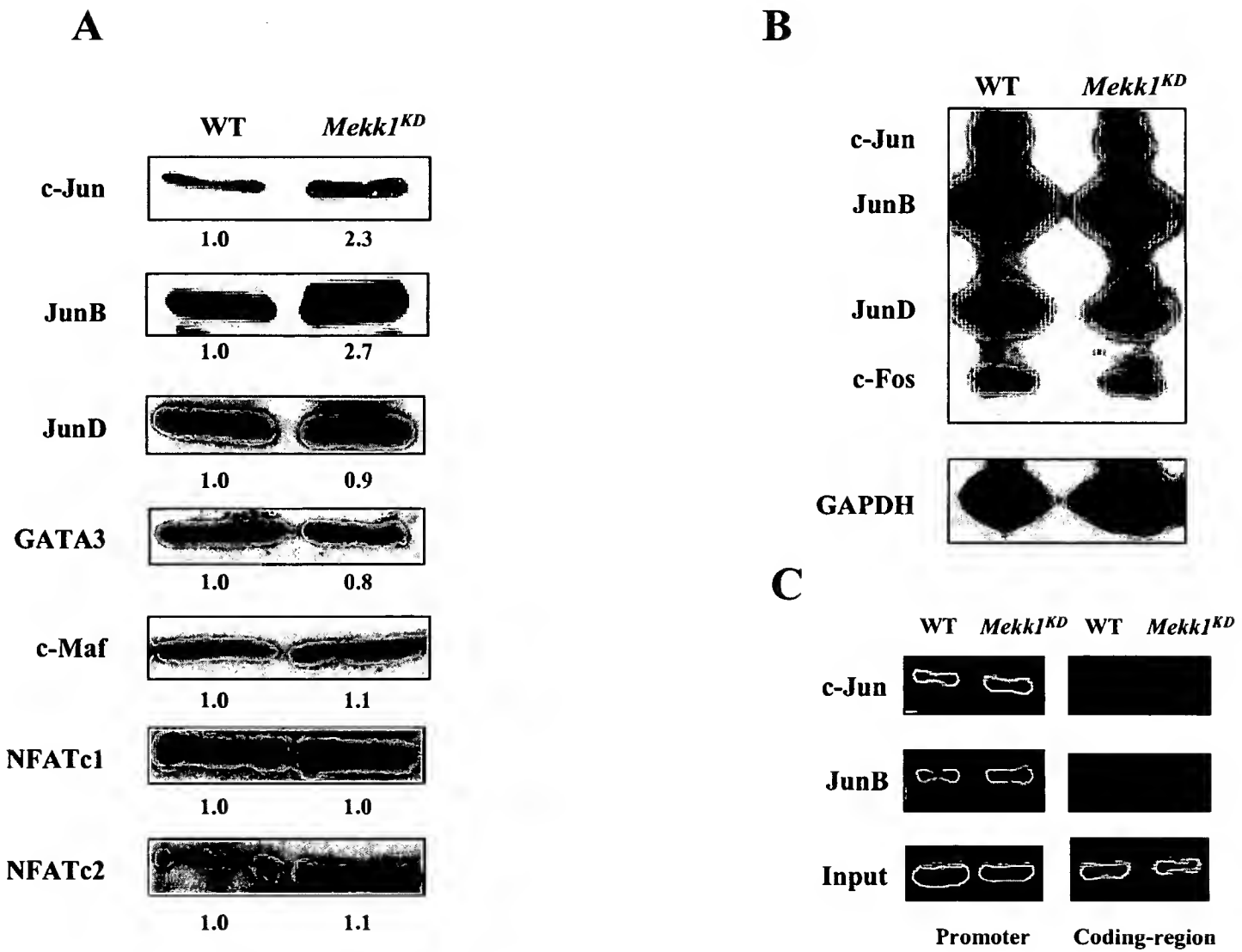
WT
Mekk1KD
IL-4
IL-13
IFNγ
L32
GAPDH
B
Relative amounts
IL-2
IL-4
IL-5
IL-10
IL-13
IFNγ
WT
Mekk1KD
C
[3H]-thymidine (cpm)
CD3 10.0 CD28 1.0
CD3 10.0
CD3 1.0
Medium
Anti-IL-4 (μg)
D
[3H]-thymidine (cpm)
IL-4 (ng)

Fig. 3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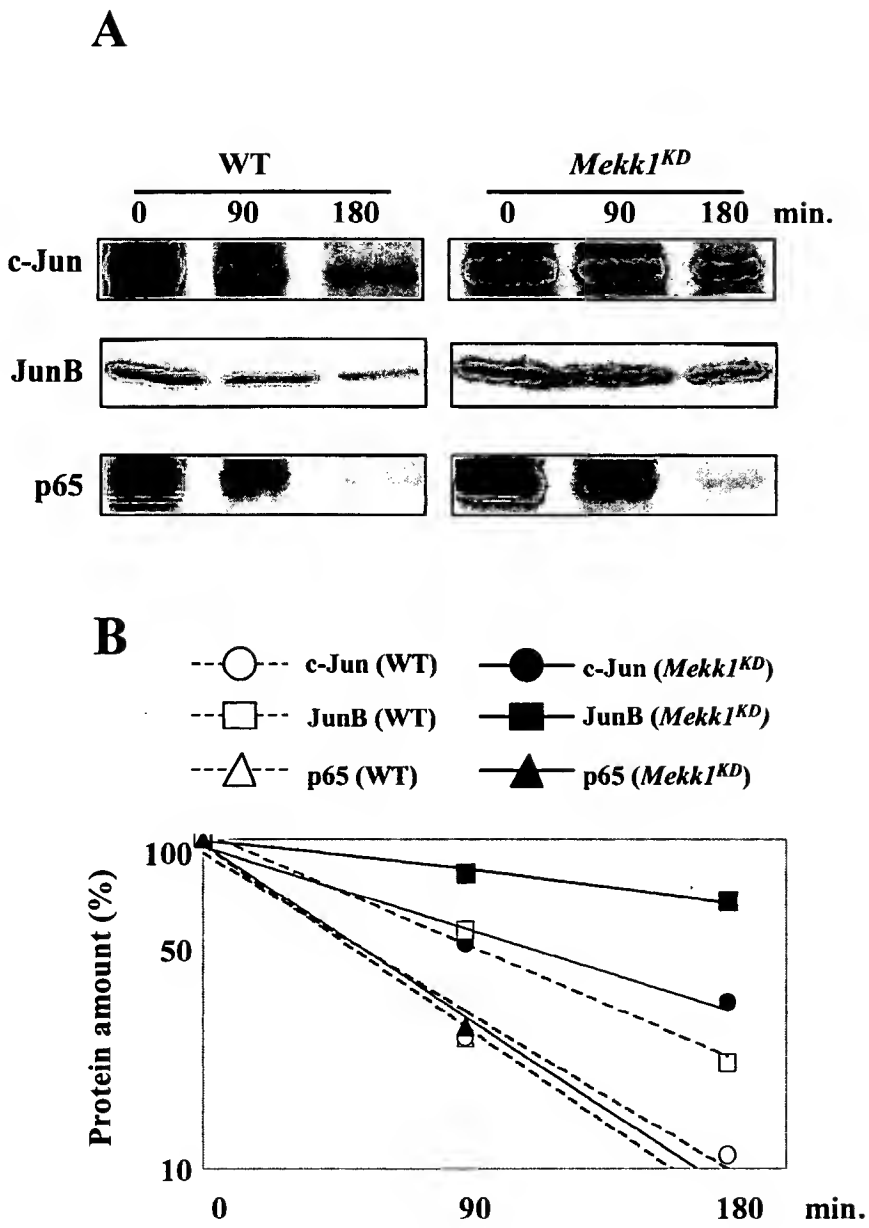
c-Jun
1.0
2.3
JunB
1.0
2.7
JunD
1.0
0.9
GATA3
1.0
0.8
c-Maf
1.0
1.1
NFATc1
1.0
1.0
NFATc2
1.0
1.1
B
WT
Mekk1KD
c-Jun
JunB
JunD
c-Fos
GAPDH
C
WT
Mekk1KD
WT
Mekk1KD
c-Jun
JunB
Input
Promoter
Coding-region

Fig. 4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WT
Mekk1KD
0
90
180
0
90
180
min.
c-Jun
JunB
p65
B
c-Jun (WT)
c-Jun (Mekk1KD)
JunB (WT)
JunB (Mekk1KD)
p65 (WT)
p65 (Mekk1KD)
Protein amount (%)
100
50
10
0
90
180
min.
C
JunB + + +
MEKK1 - + -
MEKK1 (mt) - - +
Myc-Ub + + +
Ub-JunB
JunB

Fig. 5

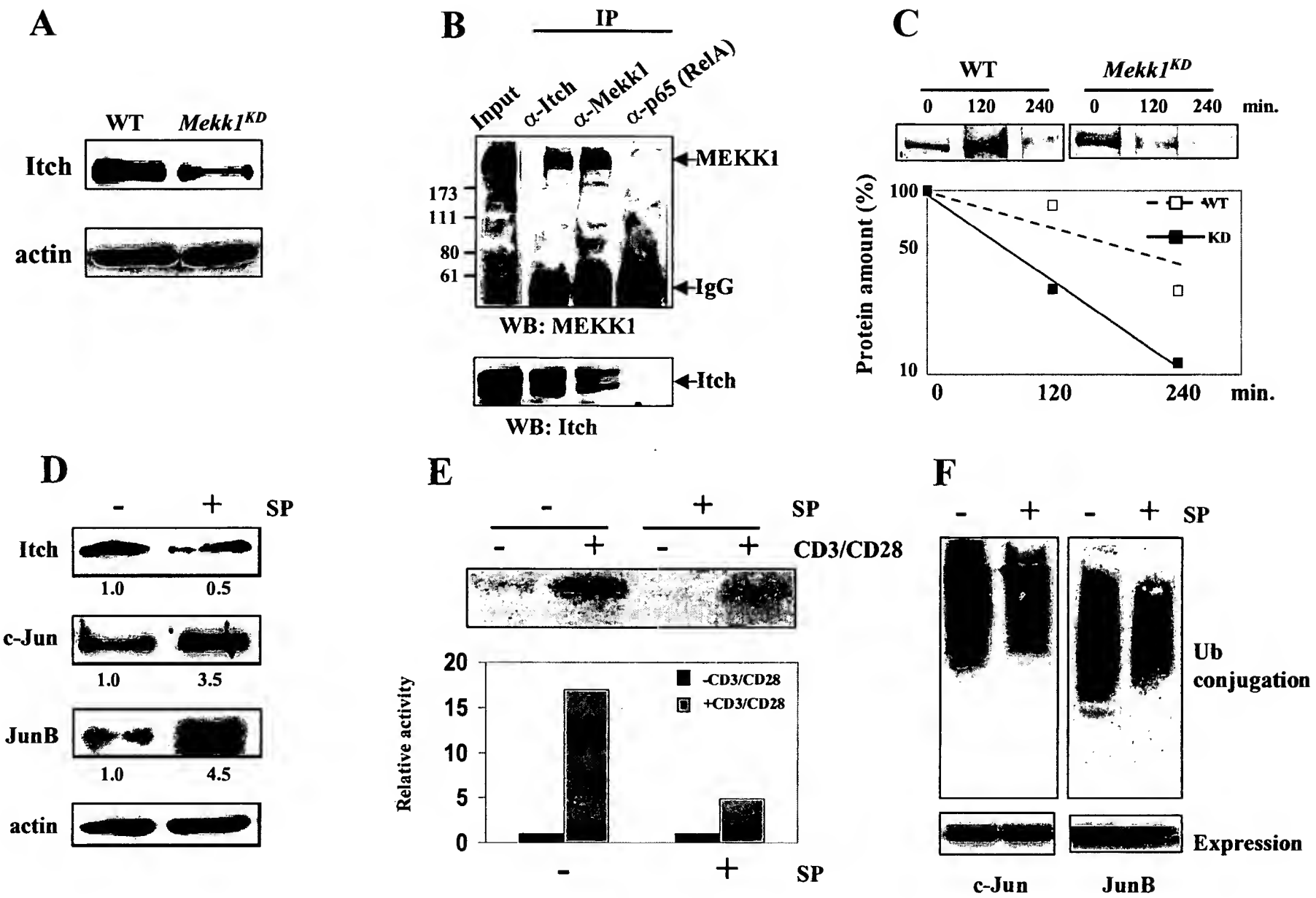

A
WT
Mekk1KD
Itch
actin
B
IP
Input
α-Itch
α-Mekk1
α-p65 (RelA)
MEKK1
IgG
173
111
80
61
WB: MEKK1
Itch
WB: Itch
C
WT
Mekk1KD
0
120
240
min.
Protein amount (%)
100
50
10
WT
KD
D
SP
Itch
1.0
0.5
c-Jun
1.0
3.5
JunB
1.0
4.5
actin
E
SP
CD3/CD28
Relative activity
20
15
10
5
0
-CD3/CD28
+CD3/CD28
F
SP
Ub conjugation
Expression
c-Jun
JunB

Fig. 6

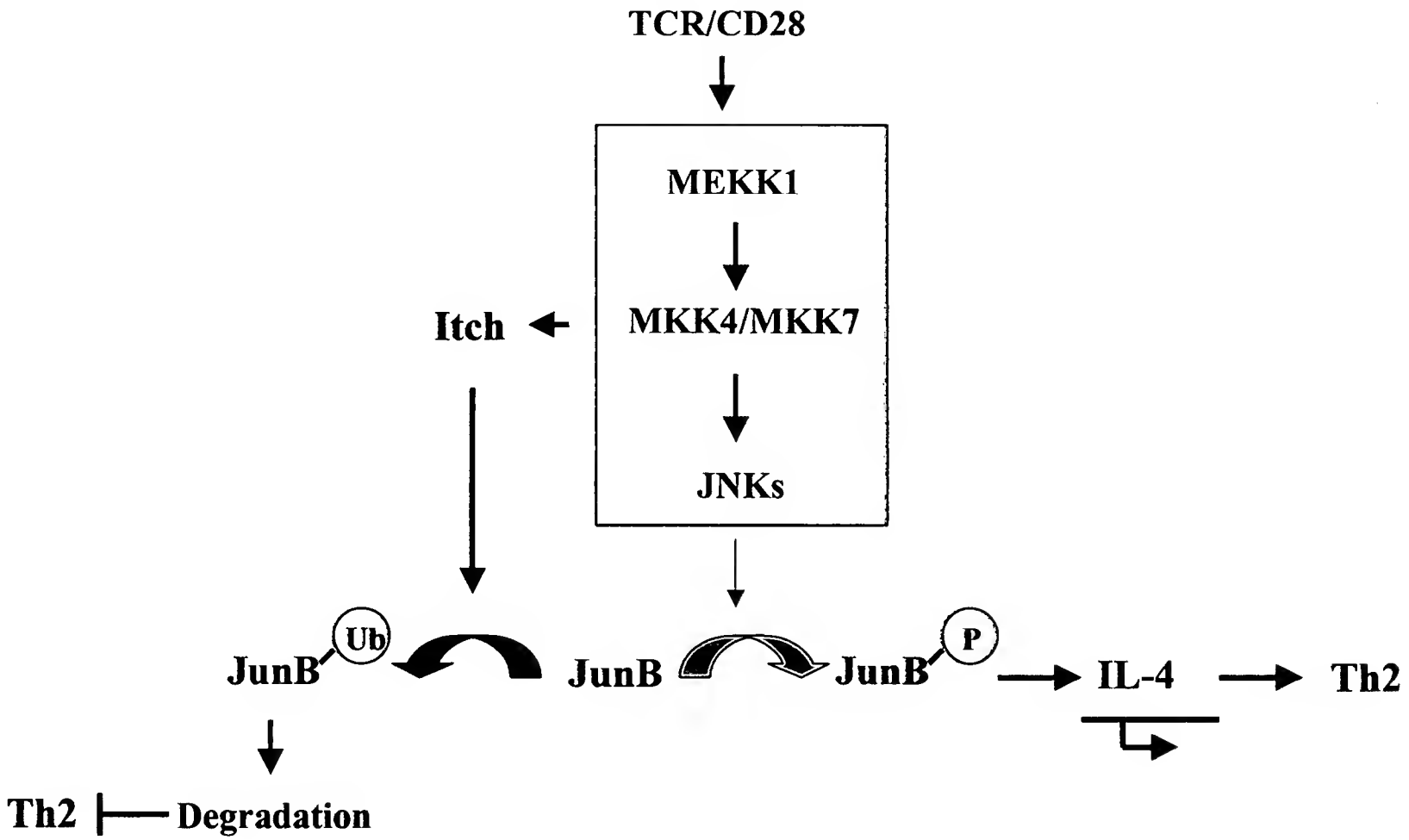

TCR/CD28
MEKK1
MKK4/MKK7
JNKs
Itch
JunB
Ub
JunB
JunB
P
IL-4
Th2
Th2
Degradation

Fig. 7

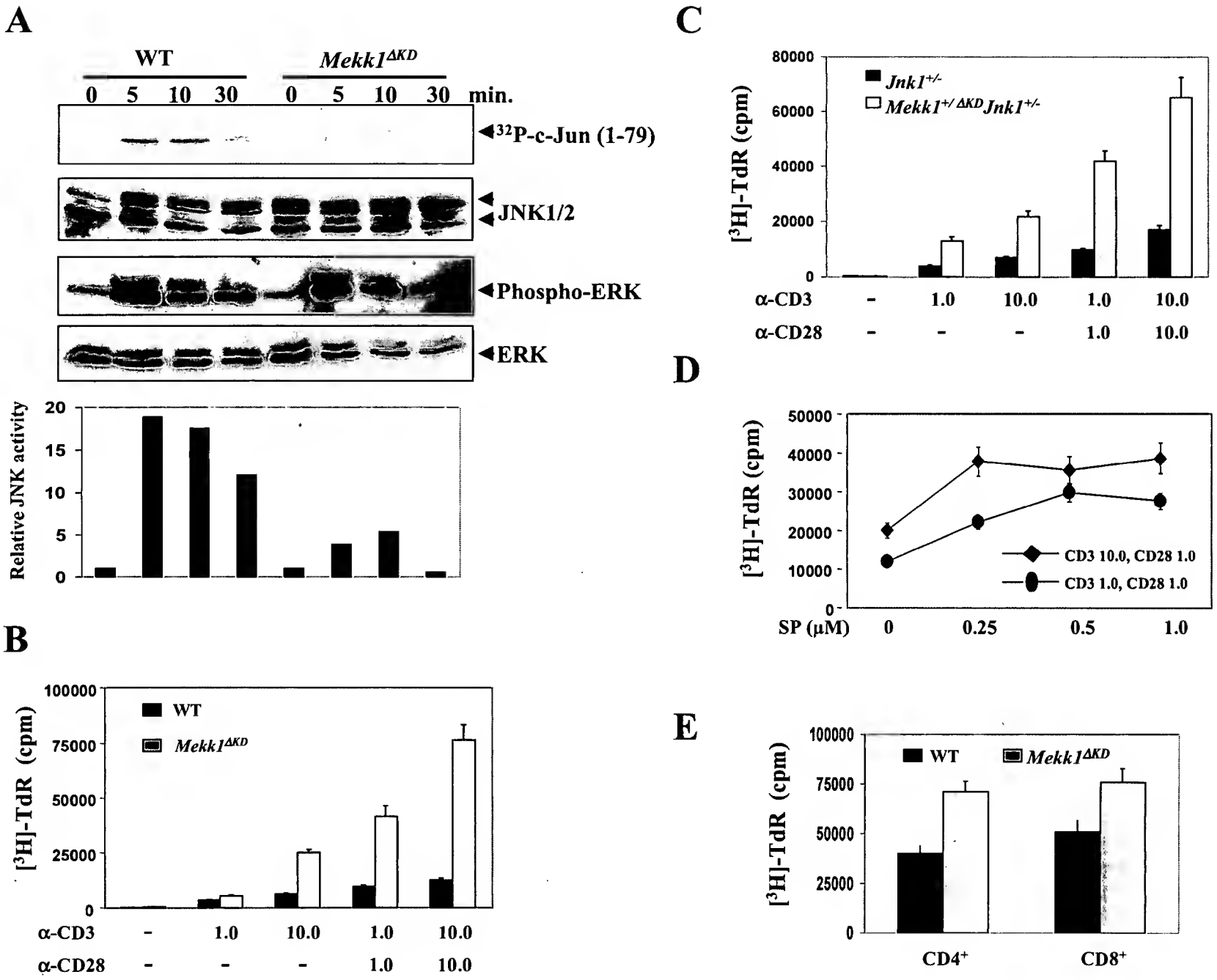

A
WT
Mekk1ΔKD
0 5 10 30 0 5 10 30 min.
32P-c-Jun (1-79)
JNK1/2
Phospho-ERK
ERK
Relative JNK activity
20
15
10
5
0
B
[3H]-TdR (cpm)
100000
75000
50000
25000
0
WT
Mekk1ΔKD
α-CD3 - 1.0 10.0 1.0 10.0
α-CD28 - - - 1.0 10.0
C
[3H]-TdR (cpm)
80000
60000
40000
20000
0
Jnk1+/-
Mekk1+/ΔKD Jnk1+/-
α-CD3 - 1.0 10.0 1.0 10.0
α-CD28 - - - 1.0 10.0
D
[3H]-TdR (cpm)
50000
40000
30000
20000
10000
0
CD3 10.0, CD28 1.0
CD3 1.0, CD28 1.0
SP (μM) 0 0.25 0.5 1.0
E
[3H]-TdR (cpm)
100000
75000
50000
25000
0
WT
Mekk1ΔKD
CD4+
CD8+

Fig. 1

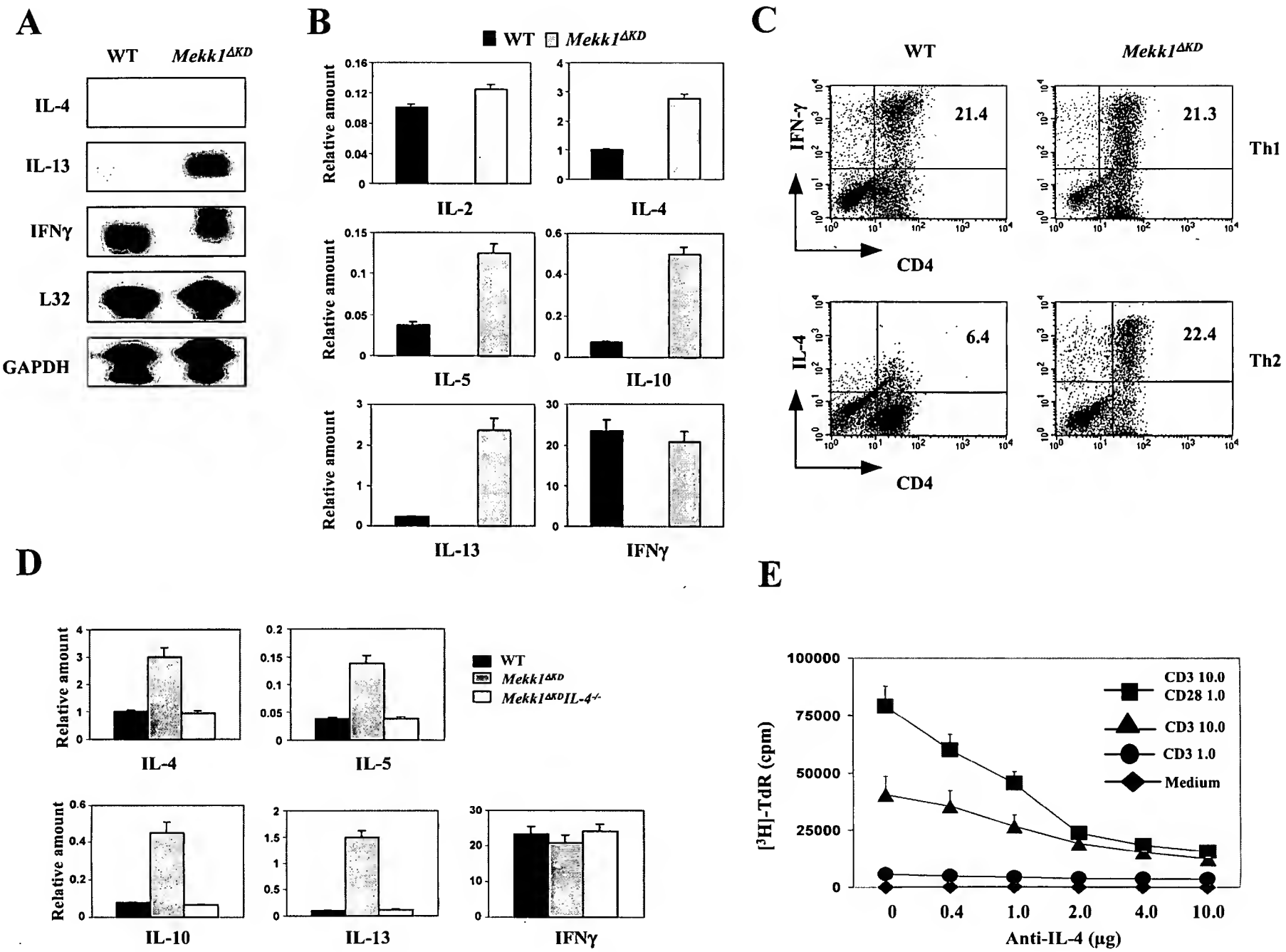
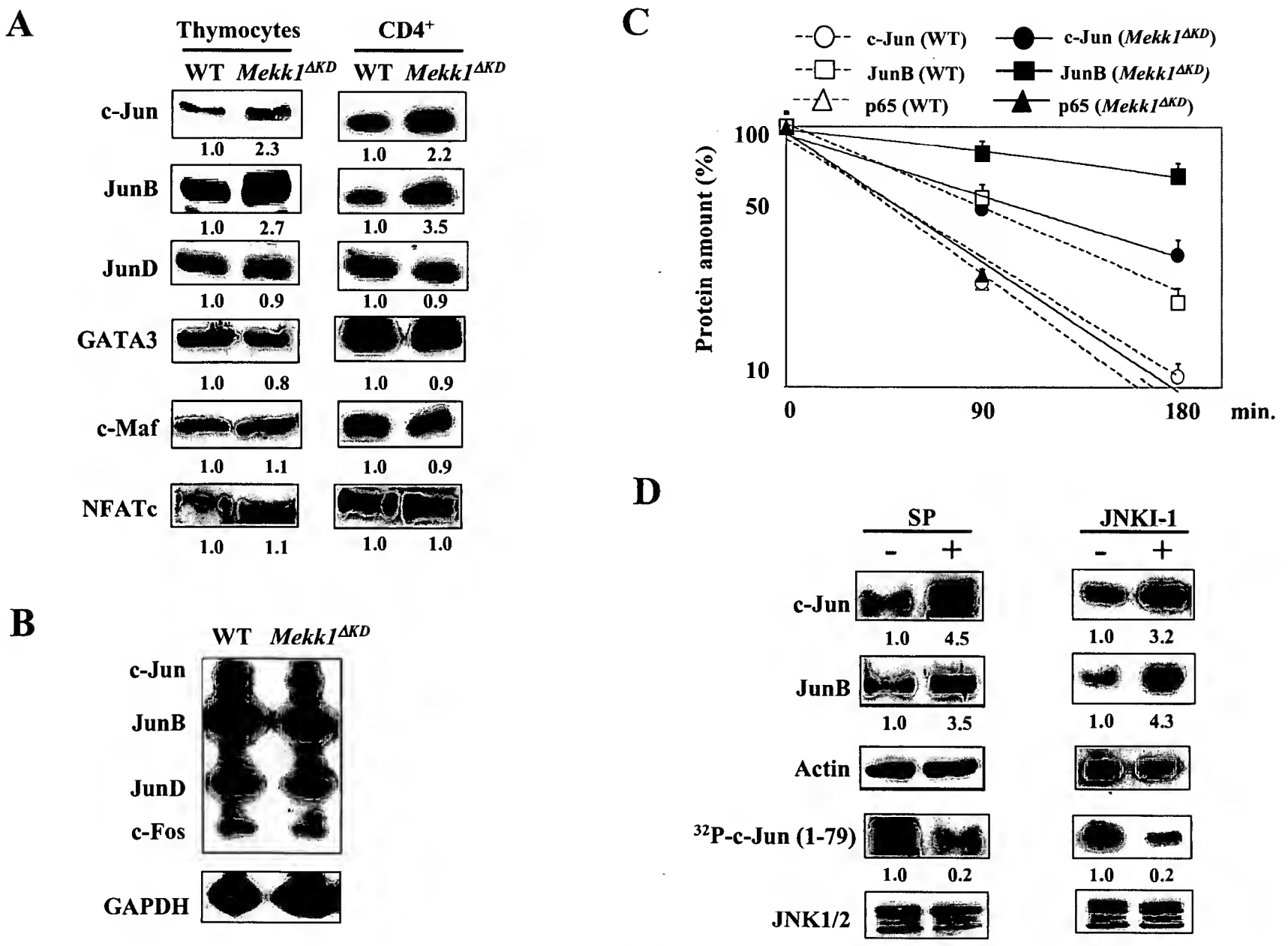

A
WT
Mekk1ΔKD
IL-4
IL-13
IFNγ
L32
GAPDH
B
WT
Mekk1ΔKD
Relative amount
IL-2
IL-4
IL-5
IL-10
IL-13
IFNγ
C
WT
Mekk1ΔKD
IFN-γ
21.4
21.3
Th1
CD4
IL-4
6.4
22.4
Th2
CD4
D
Relative amount
IL-4
IL-5
IL-10
IL-13
IFNγ
WT
Mekk1ΔKD
Mekk1ΔKDIL-4-/-
E
[3H]-TdR (cpm)
100000
75000
50000
25000
0
CD3 10.0
CD28 1.0
CD3 10.0
CD3 1.0
Medium
0
0.4
1.0
2.0
4.0
10.0
Anti-IL-4 (μg)

Fig. 2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Thymocytes
CD4+
WT
Mekk1ΔKD
WT
Mekk1ΔKD
c-Jun
1.0 2.3 1.0 2.2
JunB
1.0 2.7 1.0 3.5
JunD
1.0 0.9 1.0 0.9
GATA3
1.0 0.8 1.0 0.9
c-Maf
1.0 1.1 1.0 0.9
NFATc
1.0 1.1 1.0 1.0
B
WT
Mekk1ΔKD
c-Jun
JunB
JunD
c-Fos
GAPDH
C
c-Jun (WT)
c-Jun (Mekk1ΔKD)
JunB (WT)
JunB (Mekk1ΔKD)
p65 (WT)
p65 (Mekk1ΔKD)
Protein amount (%)
100
50
10
0
90
180
min.
D
SP
JNKI-1
-
+
-
+
c-Jun
1.0 4.5 1.0 3.2
JunB
1.0 3.5 1.0 4.3
Actin
32P-c-Jun (1-79)
1.0 0.2 1.0 0.2
JNK1/2

Fig. 3

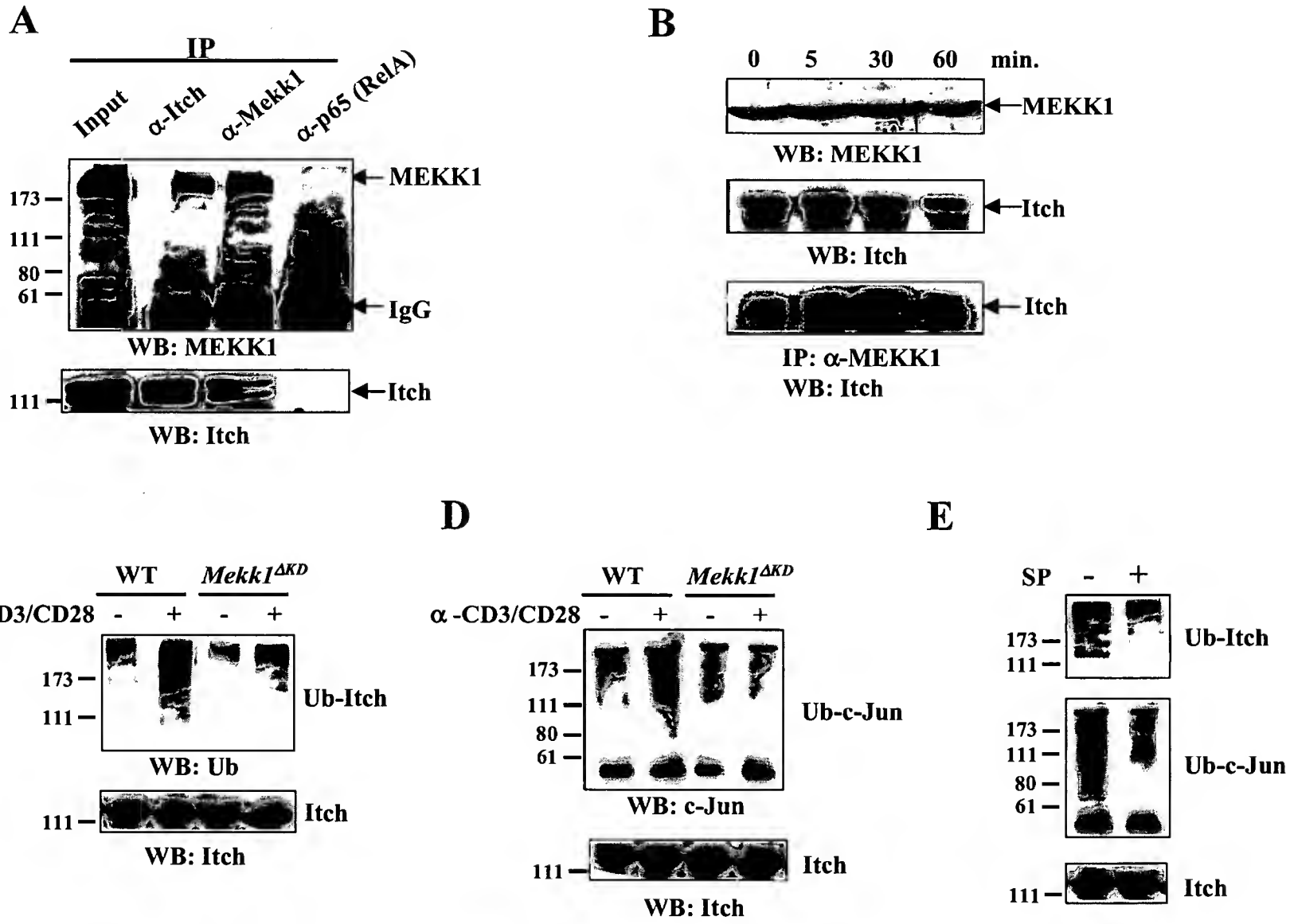

A
IP
Input
α-Itch
α-Mekk1
α-p65 (RelA)
173
111
80
61
MEKK1
IgG
WB: MEKK1
111
Itch
WB: Itch
B
0
5
30
60
min.
MEKK1
WB: MEKK1
Itch
WB: Itch
Itch
IP: α-MEKK1
WB: Itch
C
WT
Mekk1ΔKD
α-CD3/CD28
-
+
-
+
173
111
Ub-Itch
WB: Ub
111
Itch
WB: Itch
D
WT
Mekk1ΔKD
α-CD3/CD28
-
+
-
+
173
111
80
61
Ub-c-Jun
WB: c-Jun
111
Itch
WB: Itch
E
SP
-
+
173
111
Ub-Itch
173
111
80
61
Ub-c-Jun
111
Itch

Fig. 4

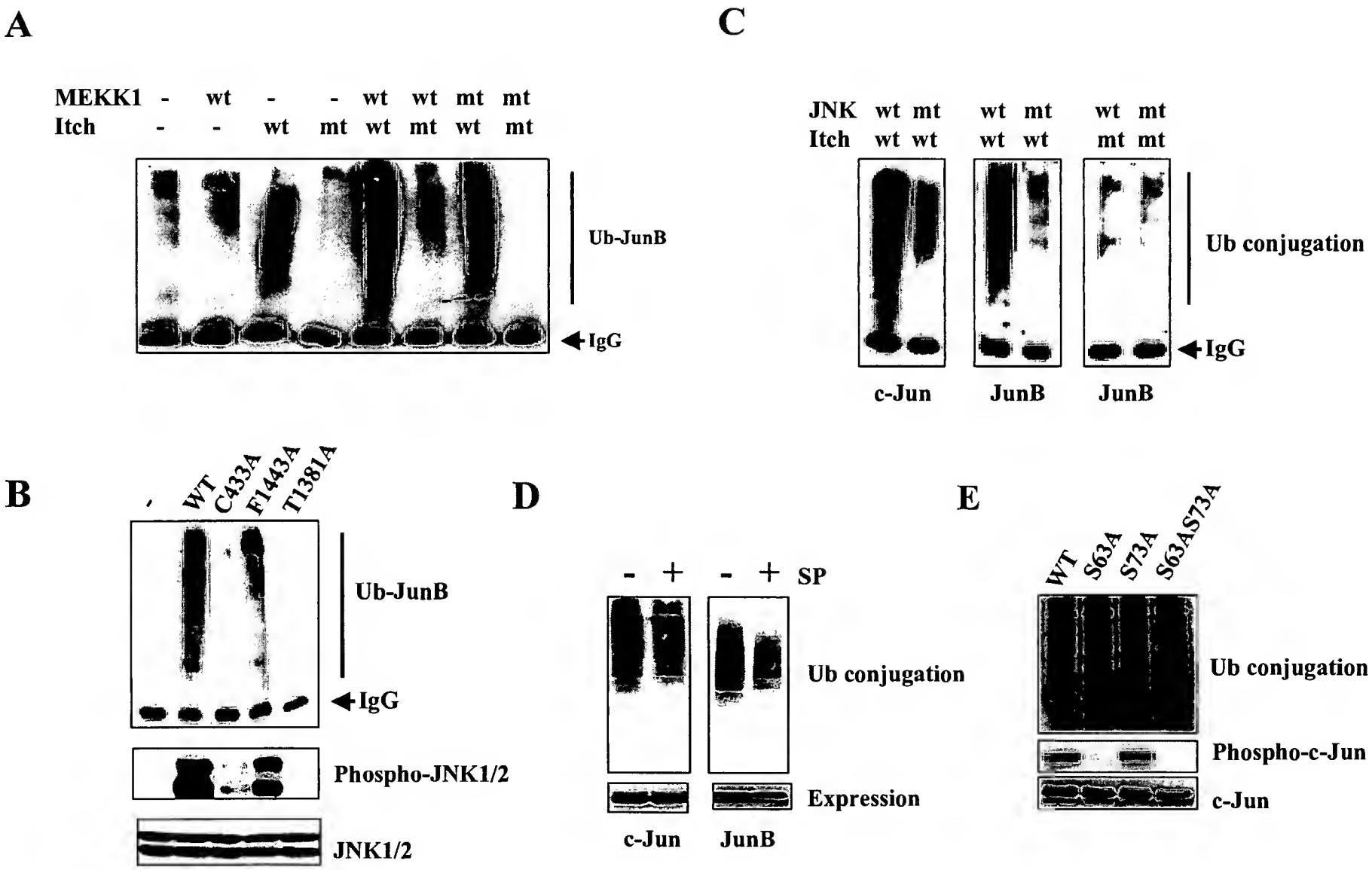

A
MEKK1 - wt - - wt wt mt mt
Itch - - wt mt wt mt wt mt
Ub-JunB
IgG
C
JNK wt mt wt mt wt mt
Itch wt wt wt wt mt mt
Ub conjugation
IgG
c-Jun
JunB
JunB
B
- WT C433A F1443A T1381A
Ub-JunB
IgG
Phospho-JNK1/2
JNK1/2
D
- + - + SP
Ub conjugation
Expression
c-Jun
JunB
E
WT S63A S73A S63AS73A
Ub conjugation
Phospho-c-Jun
c-Jun

Fig. 5

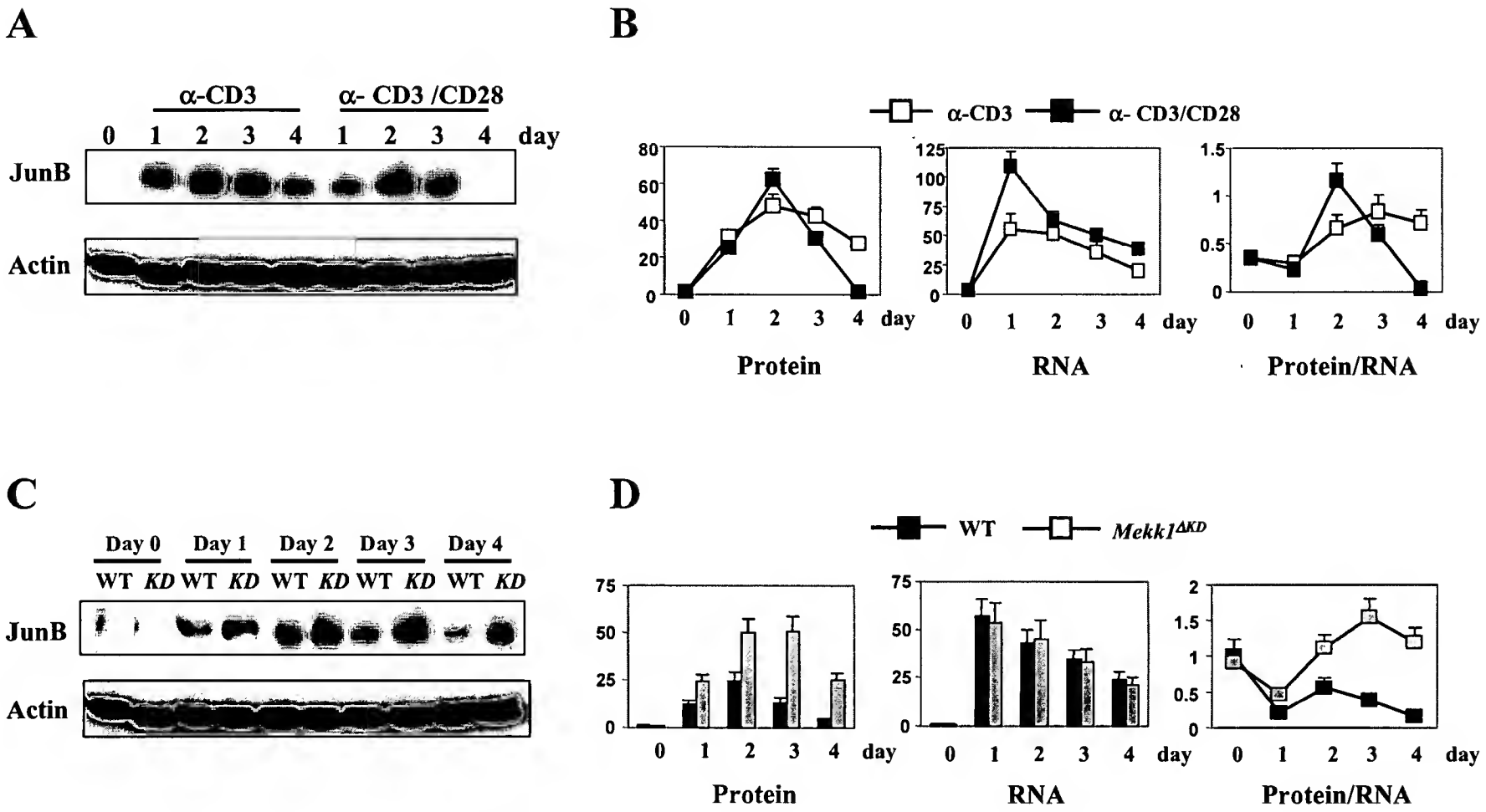
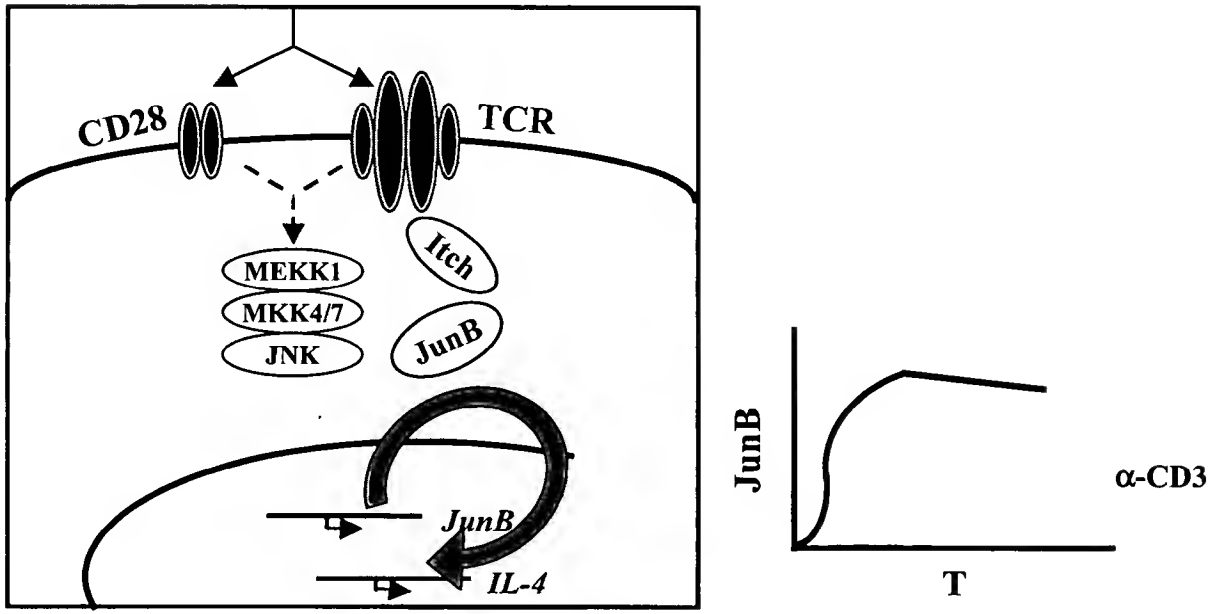

A
α-CD3
α- CD3 /CD28
0 1 2 3 4 1 2 3 4 day
JunB
Actin
B
α-CD3
α- CD3/CD28
Protein
RNA
Protein/RNA
day
C
Day 0
Day 1
Day 2
Day 3
Day 4
WT KD WT KD WT KD WT KD WT KD
JunB
Actin
D
WT
Mekk1ΔKD
Protein
RNA
Protein/RNA
day

Fig. 6

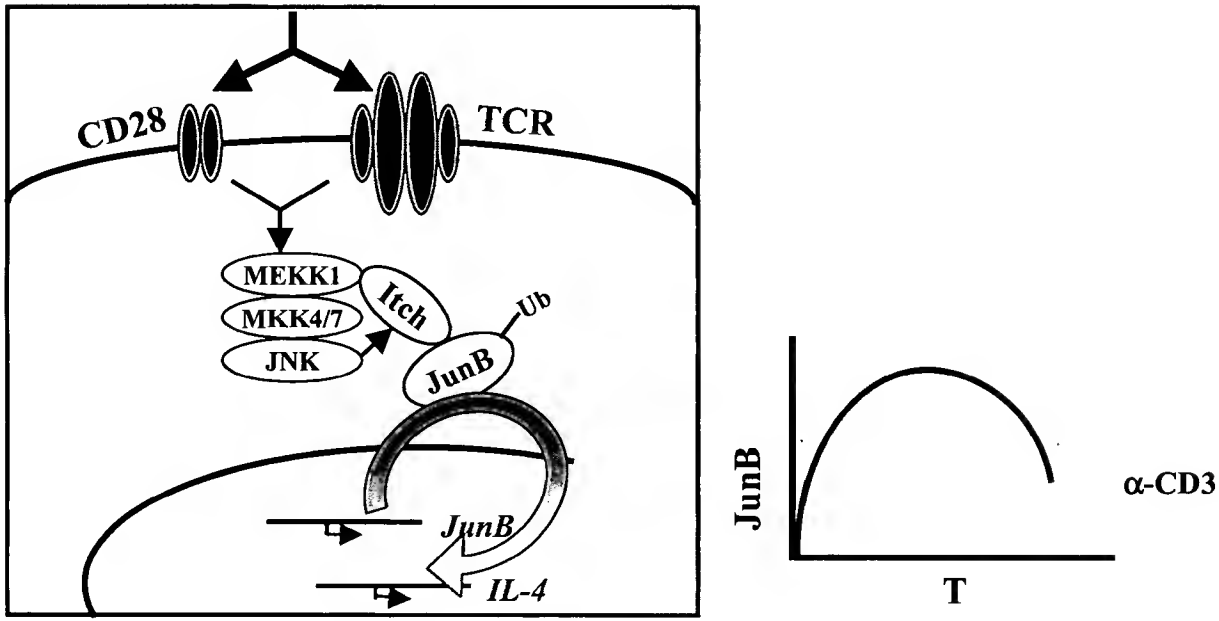
A


CD28
TCR
MEKK1
MKK4/7
JNK
Itch
JunB
JunB
IL-4

JunB
T
α-CD3

Fig. 7

B


CD28
TCR
MEKK1
MKK4/7
JNK
Itch
Ub
JunB
JunB
IL-4

JunB
T
α-CD3 + α-CD28

DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name. I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled **Enhancement of Th2-Dependent and Inflammatory Response**, the specification of which is attached hereto. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Sole or First Inventor: **Michael Karin**

Inventor's Signature: _____ Date: _____
Residence: 7710 E. Roseland Drive, La Jolla, CA 92037 Citizenship: United States of America
Post Office Address: 7710 E. Roseland Drive, La Jolla, CA 92037

Full Name of Second Joint Inventor: **Min Gao**

Inventor's Signature: _____ Date: _____
Residence: 8223 Jade Coast Road, #119, San Diego, CA 92126 Citizenship: P.R. China
Post Office Address: 8223 Jade Coast Road, #119, San Diego, CA 92126

Full Name of Third Joint Inventor: **Tord Labuda**

Inventor's Signature: _____ Date: _____
Residence: Sodra Forstadsgatan 19A, 21143 Malmo, Sweden Citizenship: Sweden
Post Office Address: Sodra Forstadsgatan 19A, 21143 Malmo, Sweden

PATENT
Attorney Docket No.: **UCSD-08805**

ASSIGNMENT

WHEREAS, WE, Michael Karin, Min Gao and Tord Labuda, hereinafter referred to as "ASSIGNOR", have invented certain new and useful improvements as described and set forth in the below-identified application for United States Letters Patent:

Title of Invention: **Enhancement of Th2-Dependent and Inflammatory Response**

Filing Date: Serial No.:

WHEREAS, The Regents of the University of California, a nonprofit California corporation, 1111 Franklin Street, 5th Floor, Oakland, CA 94607-5200, hereinafter referred to as "ASSIGNEE", is desirous of acquiring the entire right, title and interest in said invention and application and in any Letters Patent which may be granted on the same;

NOW THEREFORE, TO ALL WHOM IT MAY CONCERN: Be it known that, for and in consideration of the sum of One Dollar ($1.00) lawful money paid to ASSIGNOR by ASSIGNEE and other good and valuable consideration, receipt of which is hereby acknowledged, ASSIGNOR has sold, assigned and transferred, and by these presents does sell, assign and transfer unto said ASSIGNEE, and ASSIGNEE's successors and assigns, all right, title and interest in and to said invention, said application for United States Letters Patent, and any Letters Patent which may hereafter be granted on the same in the United States and all countries throughout the world including any divisions, renewals, continuations in whole or in part, substitutions, conversions, reissues, prolongations or extensions thereof, said interest to be held and enjoyed by said ASSIGNEE as fully and exclusively as it would have been held and enjoyed by said ASSIGNOR had this assignment and transfer not been made, to the full end and term of any Letters Patent.

ASSIGNOR further agrees that ASSIGNOR will, without charge to said ASSIGNEE, but at ASSIGNEE's expense, cooperate with ASSIGNEE in the prosecution of said application and/or applications; execute, verify, acknowledge and deliver all such further papers, including applications for Letters Patent and for the reissue thereof, and instruments of assignment and transfer thereof; and will perform such other acts as ASSIGNEE lawfully may request, to obtain or maintain Letters Patent for said invention and improvement in any and all countries, and to vest title thereto in said ASSIGNEE, or ASSIGNEE's successors and assigns.

IN TESTIMONY WHEREOF, ASSIGNOR has hereunto signed ASSIGNOR's names to this assignment on the date indicated below.

_____ _____

Michael Karin Min Gao

_____

Tord Labuda

STATE OF _____)
) ss.
COUNTY OF _____)

On this _____ day of _____, in the year of _____, before me, the undersigned notary public, personally appeared the above-named ASSIGNOR, known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument, and acknowledged that he/she executed the same.

_____

NOTARY PUBLIC

SEAL

PATENT
Attorney Docket No.: **UCSD-08805**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Michael Karin *et al.* Group No.:
Serial No.: Examiner:
Filed:
Entitled: **Enhancement of Th2-Dependent and Inflammatory Response**

POWER OF ATTORNEY BY ASSIGNEE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

The Regents of the University of California, as Assignee of record of the entire interest of the above-identified patent application, hereby appoints the members of the firm of MEDLEN & CARROLL, LLP, a firm composed of:

Peter G. Carroll	(Reg. No. 32,837)	David A. Casimir	(Reg. No. 42,395)
Tanya A. Arenson	(Reg. No. 47,391)	Maha A. Hamdan	(Reg. No. 43,655)
Jason R. Bond	(Reg. No. 45,439)	Thomas C. Howerton	(Reg. No. 48,650)
Mary Ann D. Brow	(Reg. No. 42,363)	J. Mitchell Jones	(Reg. No. 44,174)
Thomas W. Brown	(Reg. No. 50,002)	Christine A. Lekutis	(Reg. No. 51,934)
Robert A. Goetz	(Reg. No. P-55,210)	David J. Wilson	(Reg. No. 45,225)

as its attorneys with full power of substitution to prosecute this application and transact all business in the Patent and Trademark Office in connection therewith.

Please direct all future correspondence and telephone calls regarding this application to:

Maha A. Hamdan
MEDLEN & CARROLL, LLP
101 Howard Street, Suite 350 Telephone: 415/904-6500
San Francisco, California 94105 Facsimile: 415/904-6510

I hereby certify that the Assignment document filed with the application or filed subsequent to the filing date of the application, has been reviewed and I hereby certify that, to the best of my knowledge and belief, title is with The Regents of the University of California.

Dated: _____ By: _____

Name: _____

Title: _____

The Regents of the University of California
1111 Franklin Street, 5th Floor
Oakland, CA 94607-5200